Exploiting 3D printing and digital imaging technology to develop affordable phenotypic assays for rapid and portable detection of bacterial antibiotic resistance

A Thesis Submitted to the University of Reading in Partial Fulfilment for the Degree of Doctor of Philosophy

by

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AUTHOR'S DECLARATION

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

Diep The Tai

ACKNOWLEDGEMENTS

It is my great pleasure to acknowledge and thank the University of Reading for supporting my fellowship to study and live in the UK. I would also like to thank the Pasteur Institute in Ho Chi Minh city and my colleagues for supporting my study.

I would like to express my sincere gratitude to my supervisors Dr. Partha Pratim Ray and Dr. Samuel Bizley who gave many valuable comments and suggestions for my thesis, and a special thanks to Dr. Alexander Daniel Edwards who gave me the opportunity to come and do my thesis with him. Without his guidance and enthusiasm, this dissertation wouldn't have been possible.

My sincere thanks also goes to Sarah Needs, Sultan Ilayda Donmez, Ruya Meltem Sariyer, and Matthew Long as well as all my Vietnamese friends for their support and encouraging me. I would also like to thank Barney Jones who collected milk samples for me, and George Gibbings who prepared sterilized media for me at the Agricultural Department. I appreciate all support from the laboratory technicians of the Food laboratory who helped me prepare sterile media on time.

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Last but not least, I am extremely grateful to my family, especially my mom for their love and sacrifices for my study**.**

Content

ABSTRACT

Rising antimicrobial resistance (AMR) is a challenge to public health, animal health, and the environment. Early detection of phenotypic-based antimicrobial resistance remains an essential step to effectively optimizing antibiotic treatments. By using 3D printed technology and Raspberry Pi - computers, two novel assays were developed for early detection of antimicrobial resistance based on motility and the bacterial response to antibiotics on agar media. Integrating culture steps and determination of minimum inhibitory concentration (MIC) into one single test, this thesis elucidated the identification of bacterial species and their MIC within hours using a frame dip slide, as well as within just 5 minutes of testing to show the results of bacterial susceptibility based on their movement or stop swimming.

In addition, the temperature remains a crucial factor for bacterial growth. By designing a mobile incubator, all microbiology tests can be carried out in a variety of settings including next to the bed of patients or in the field, instead of in the laboratory. Automation of image analysis contributes another tool to better understand physiological bacteria when living in vitro conditions. These successful tools contributed alternative approaches to surveying at real-time detection of antimicrobial resistance in the field.

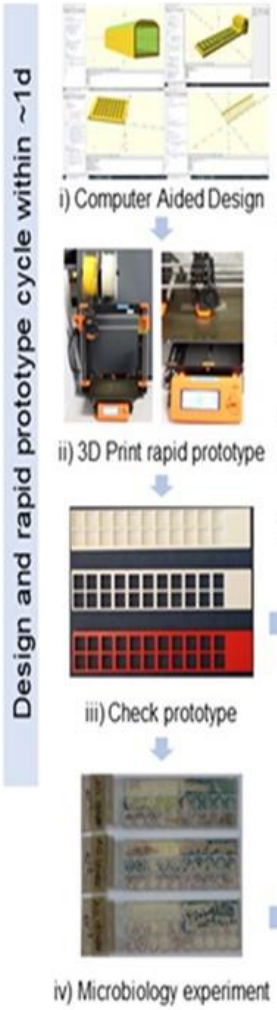
Beyond the laboratory, these tools brought the concept to combine all-in-one. Using a frame dip slide combined with a mobile incubator as well as combining Microcapillary Film (MCF) with 3D printed microscopy created a portable kit for affordable phenotypic assays for rapid detection of bacterial antibiotic resistance anywhere, not just for performing in the laboratory.

DETAILS OF PUBLICATIONS SUPPORTING THE PhD

Mobile incubator

MicroMI: A portable microbiological mobile incubator that uses inexpensive lithium power banks for field microbiology (2021). HardwareX.

3D print

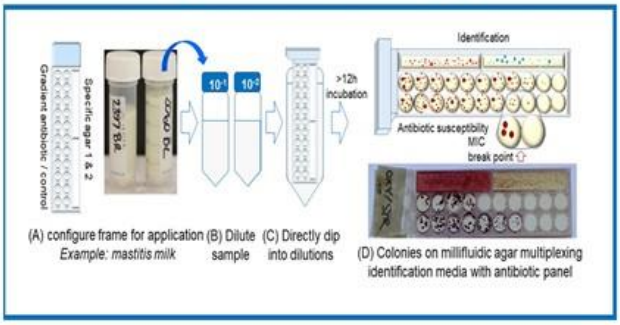


Methods for rapid prototyping novel labware: using CAD and desktop 3D printing in the microbiology laboratory (2021). Letters in Applied Microbiology

Automate time-lapse image

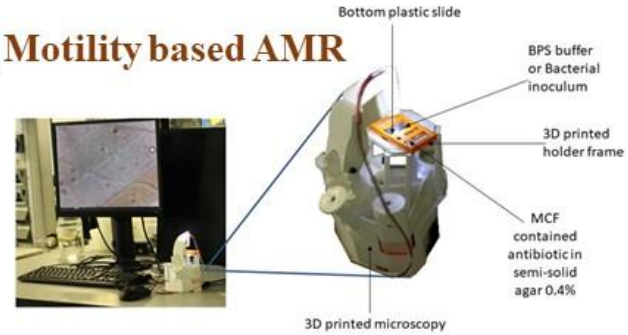
Exploiting open-source 3D printer architecture for laboratory robotics to automate high-throughput time-lapse imaging for analytical microbiology (2019). Plos one.

Frame dip slide – phenotypic AMR



3D-Printed Dip Slides Miniaturize Bacterial Identification and Antibiotic Susceptibility Tests Allowing Direct Mastitis Sample Analysis (2022). Micromachines

Motility based AMR



(2022). Micromachines

Figure A: The publications supporting this Ph.D. thesis. The brown words show the topic of work, the black words show the name of the paper and the year of publication, and the blue words show the name of the journal where the paper was published.

Design microbiology labware - 3D print (Chapter 2 - page 38)

This study elucidated the role of 3D technologies and their promising application in microbiology.

Full paper

Tai The Diep, Partha Pratim Ray, Alexander Daniel Edwards (2021). **Methods for rapid prototyping novel labware: using CAD and desktop 3D printing in the microbiology laboratory.** Lett Appl Microbiol 2022 Feb;74(2): 247 - 257. PMID: 34826147. **https://doi.org/10.1111/lam.13615**

The extent of Contribution to research:

In this work, I:

1. Designed some consumables for the microbiology laboratory
2. Designed the experiment
3. Performed the test
4. Collected and analysed the data
5. Drafted the manuscript

Frame dip slide - phenotypic AMR (Chapter 3 - page 39)

This study showed evidence of the effectiveness of 3D frames and digital cameras in the early detection of antimicrobial resistance

Conference Abstract (Russian - Vietnamese Scientific and Practical Conference - Moscow 2019)

Tai The Diep (2019). Antimicrobial resistance on isolates from the human, environment in Southern region, Vietnam. **Oral Presentation.**

Full paper

Tai The Diep, Samuel Bizley, Alexander Daniel Edwards (2022). **3D-Printed Dip Slides Miniaturize Bacterial Identification and Antibiotic Susceptibility Tests Allowing Direct Mastitis Sample Analysis.** Micromachines 2022, 13, 941. **https://doi.org/10.3390/mi13060941**

The extent of Contribution to research:

In this work, I:

1. Designed the experiment and prototype of the frame.
2. Performed the test
3. Collected and analysed the data
4. Drafted the manuscript

Mobile incubator (Chapter 4 - page 40)

This study has described the design of the mobile incubator and its application.

Full paper

Tai The Diep, Samuel Bizley, Partha Pratim Ray, Alexander Daniel Edwards (2021). **MicroMI: A portable microbiological mobile incubator that uses inexpensive lithium power banks for field microbiology.** HardwareX 10 (2021) e00242. https://doi.org/10.1016/j.ohx.2021.e00242

The extent of Contribution to research:

In this work, I:

1. Designed the experiment
2. Performed the test
3. Collected and analysed the data
4. Drafted the manuscript

Motility-based AMR (Chapter 5 - page 41)

This study described the application of 3D microscopy combined with a Raspberry Pi camera and MCF for early detection of antimicrobial resistance

Conference Abstract (**The 23rd International Conference on Miniaturized Systems for Chemistry and Life Sciences (µTAS 2019) -** Basel, SWITZERLAND

Tai The Diep, Alexander Daniel Edwards (2019). 3D printed Raspberry Pi Microscopy for low-cost Microfluidic bacterial motility analysis**. Poster presented.**

Full paper

Tai The Diep, Samuel Bizley, Alexander Daniel Edwards (2022). **Integrating 3D printed microscopy with advanced MCF technology to detect bacterial motility: an approach to rapid detection of antimicrobial resistance.** This work is planned to publish in Micromachines Journal.

The extent of Contribution to research

In this work, I:

1. Printed a 3D microscope
2. Designed the experiments
3. Performed the test
4. Collected and analysed the data
5. Drafted the manuscript

Automate time-lapse imaging (Chapter 6 - page 44)

This study described the application of 3D architecture for laboratory robotic

Conference Abstract (54th US-Japan Cholera Conference in Osaka - 2019)

Diep The Tai, Sarah Needs, Alexander Daniel Edward (2019). Open-source hardware: Automated time-lapse for visualizing bacterial colony and sugar utilization with POLIR. **Poster presented.**

Full paper

Sarah H. Needs, Tai The Diep, Stephanie P. Bull, Anton Lindley-Decaire, Partha Ray, Alexander D. Edwards (2019). **Exploiting open-source 3D printer architecture for laboratory robotics to automate high-throughput time-lapse imaging for analytical microbiology.** PLoS One. 2019 Nov 9;14(11):e0224878. PMID: 31743346. https://doi.org/10.1371/journal.pone.0224878

The extent of Contribution to research

In this work, I:

1. Performed the microbiology test
2. Wrote the methods for the microbiology test
3. Collected and analysed the data for the microbiology section

RESEARCH AIMS - HYPOTHESIS - OBJECTIVES

Aims

- To develop mobile portable equipment and alternative methods for the rapid detection of antimicrobial resistance - bacterial susceptibility testing.

Hypothesis - questions

- How to create the labware using 3D printing
- How to develop phenotypic-based methods for the rapid detection of AMR combined with 3D printing.
- How to move a microbiological incubator outside of the lab
- How to measure antibiotic susceptibility with motility

Objectives:

- Create labware tools for a microbiology laboratory by using OpenSCAD and 3D printing.
- Design a frame dip slides to culture and determine MIC breakpoints for bacteria.
- Design a mobile incubator using Uninterruptible Power Supply (UPS) instead of mains electricity.
- Use 3D printed microscopy combined with Micro capillary Film (MCF) to detect bacterial susceptibility based on motility.

List of Abbreviations

AMR	Antimicrobial resistance
CDC	Control Disease Centre
CFU	Colony form unit
FDA	US Food and Drug Administration
GDB	Gross Domestic Product
GLASS	Global Antimicrobial Resistance Surveillance System
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin- susceptibility *Staphylococcus aureus*
MCF	Micro capillary Film
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
NAG	Non agglutination
PCR	Polymerase chain reaction
POC	Point of care
PVOH	Polyvinyl alcohol
TB	Tuberculosis
UPS	Uninterruptible Power Supply

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CHAPTER 1 - CONTEXTUAL

Part 1 - Introduction

1.1. Global use of antibiotics and increase in antimicrobial resistance

Antibiotics are small molecule drugs that inhibit the growth of microbes that have dramatically changed modern medicine. Before the word antibiotic was first used in 1941 (1), a variety of natural herbs were used for the treatment of human infection documented by historical evidence (2, 3). In over 100 years since the first drug - salvarsan - was used to treat *Treponema pallidum*, the causative agent of syphilis, many classes of antimicrobial agents have been developed and have contributed to ameliorating the negative impact of infections on human health (3). This kick-started the golden era of antibiotics from 1940 - to 1962 (figure 1.1) when most of the current antibiotics were discovered and launched onto the market. However, the impact of the overuse of antibiotics and human activities such as industrialization, urbanization is forcing the increase of antibiotic resistance, with the capacity of bacteria no longer respondings to the antibiotics designed to kill them, according to CDC (figure 1.2).

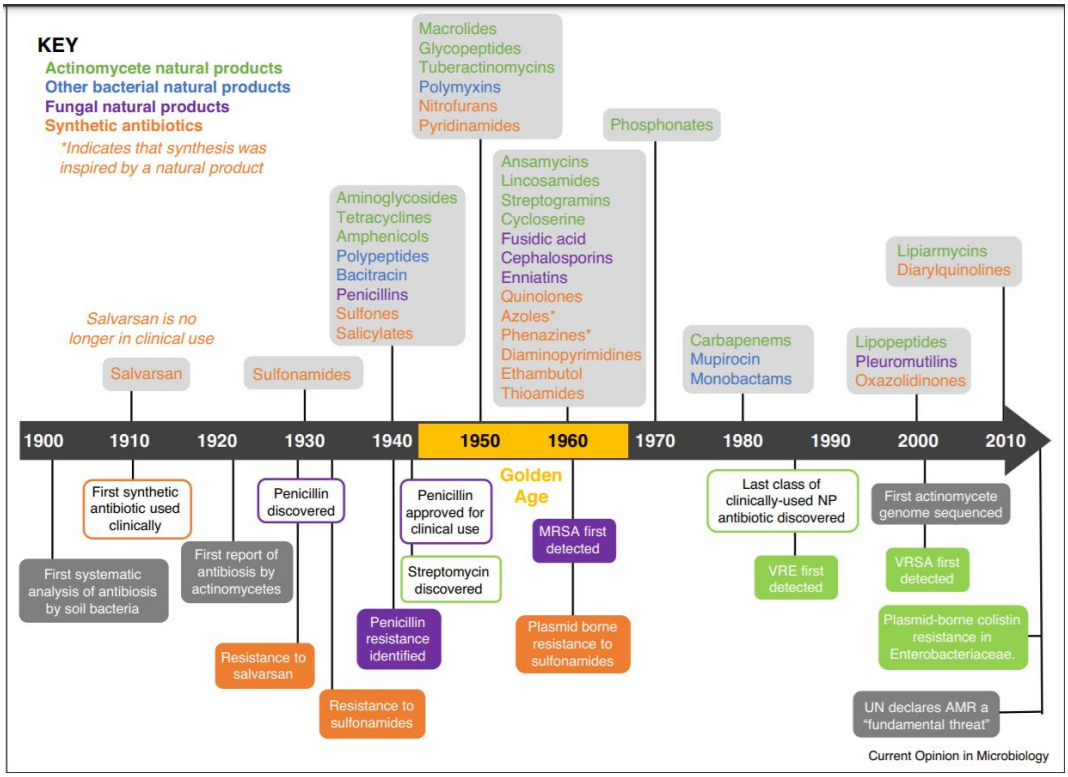

Figure 1.1: The timeline of the development of antibiotics reached the clinic. Source: Matthew (2019). Antibiotics: past, present, and future. Current Opinion in Microbiology 2019, 51:72–80.

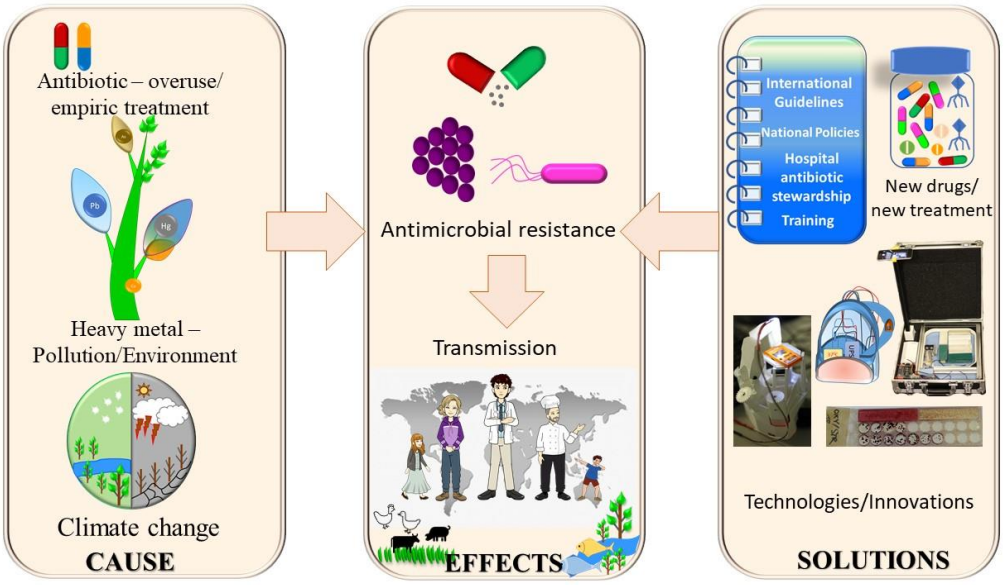

Figure 1.2: Causes of increasing antibiotic resistance and their impact on humans as well as the solutions to fighting the problem.

Antibiotic use has risen rapidly along with the population growth rate. It is estimated that antibiotic use has increased by 65% between 2000 and 2015, this increasing trend will reach 200% between 2015 and 2030 worldwide (4). Antibiotic consumption has not only increased in the human population but in the animal sector. It is estimated around 73% of antibiotics sold globally are used on animals to increase food production (5). According to World Health Organization, there are 3 major categories classified for antibiotics used in animals: therapeutic, disease prevention, and growth promotion. In the 1940s, penicillin was used for treating bovine mastitis, as well as this in 1948 sulfaquinoxaline was the first antibiotic to be administered in poultry feed to prevent coccidiosis (6). In 1950, penicillin, chlortetracycline, doxycycline, and sulfonamides were the first antibiotics approved for use in livestock by the US Food and Drug Administration (FDA) (7). Since then, there has been an increase in antibiotic use in livestock by 67% globally (2010 - 2030). Many antibiotics used in humans such as tetracycline, sulfonamides,

lincosamides, cephalosporins, and fluoroquinolones are currently used in beef, dairy, pork, and poultry industries (6). Moreover, in America, around 70% of antibiotics used to treat human infection are sold for use in animal feed (8).

The pressure on animal protein production is also another factor that increases the demand for antibiotic consumption globally. Aquatic animals supplied 17% of animal protein nutrition worldwide, for over 40% of the world's population, fish contributed nearly 20% of per capita animal protein consumed (9). The rising demand for animal protein nutrition has transferred intensively to the production systems and has led to an increase in antibiotic consumption to maintain the health and productivity of aquatic farms. It is estimated antimicrobial consumption will rise 33% by 2030 compared to 2017 in aquaculture (9) and is expected to rise by 11.5% on agricultural farms by 2030 (5). Antibiotic consumption in Asian countries was the highest compared to other regions in the world (figure 1.3).

Increasing antibiotic consumption associated with antimicrobial resistance has contributed to the global health threat and is well documented. Since 2013, the program surveillance relationship between resistance and antibiotic consumption has been deployed. The Global Antimicrobial Resistance Surveillance System (GLASS) has been collecting data from 81 countries since 2014 (4). Much considerable research has revealed a significant correlation between antimicrobial consumption and carbapenem resistance in Gram-negative bacteria isolated in inpatients, in China (2019) a 60% increase in carbapenem resistance was recorded (10). The overuse of carbapenem and cephalosporins have induced the resistance of *E.coli* and *Klebsiella pneumonia* to Ciprofloxacin, and *Acinetobacter baumanii* to Imipenem in Korea (11). Some frequently used antibiotics such as tetracycline, aminoglycoside, and penicillin groups in animal feeds were the main factor raising the multidrug-resistant isolates from 20% in Nigeria to 100% in South Africa, Zimbabwe, Tunisia (12) and greater than 80% tetracycline-resistant bacteria found in China as well as over 99% homology of apramycin resistant genes were found on *E.coli* isolated from human and animal (13). Research in

Ethiopia, Uganda, and Bangladesh has also elucidated the similarity of resistant *E.coli, Salmonella,* and *Klebsiella* isolated on humans and animals (14-16).

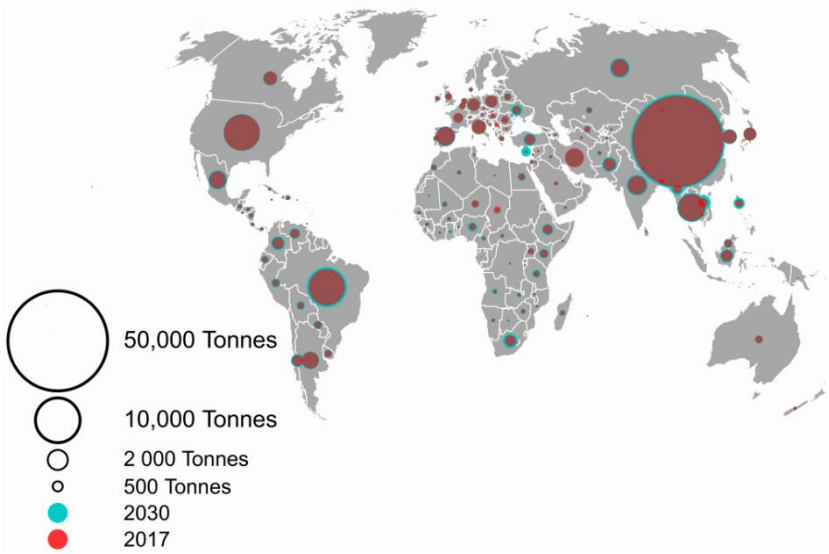

Figure 1.3: Estimated antimicrobial consumption per country in 2017 and 2030. The size of the circles corresponds to the amounts of antimicrobials used. Source: Katie Tiseo (2020). Global Trends in antimicrobial Use in Food Animals from 2017 to 2030. Antibiotics 2020, 9, 918; doi:10.3390/antibiotics9120918

Antibiotic-resistant organisms are significant contributors to morbidity and mortality globally. In 2018, antibiotic consumption according to a WHO report had dramatically increased, the overall usage was from 4.4 to 64.4 defined daily doses (DDD) per 1000 inhabitants per day. The most frequently consumed antibiotic was amoxicillin and amoxicillin/clavulanic acid in the majority of countries (17). Whilst, resistance and consumption rates varied across countries, penicillin remains the most used antibiotic in low - medium-income countries and high-income countries. Cephalosporins antibiotics increased the demand in low and medium-income countries (4). Antimicrobial pathogens such as *E. coli, Klebsiella, Acinetobacter, Staphylococcus aureus, Streptococcus pneumoniae, Salmonella, Shigella, Neisseria gonorrhoeae* have been noted to global surveillance, unfortunately, most of them have highly resisted third-generation cephalosporin, fluoroquinolones, carbapenems, and methicillin-resistant *Staphylococcus aureus* (18) and the bacterial resistance differed from high-income countries to low-income countries.

Every year, 700.000 people die of drug-resistant infections and multidrug-resistant Tuberculosis (MDR – TB) has been reported in 123 countries. It was estimated that by 2050 there will be at least 10 million deaths caused by antimicrobial resistance each year and a loss of up to 100 trillion USD from global GDB, the highest number of mortalities is estimated in Asia with 4,730,000 cases, four times higher than the estimated total number of cases in Europe, North America, Latin America, and Oceania, as equal as to several deaths in Africa (19).

Meanwhile, no new classes of antibiotics have been developed and used for treatment since the 1980s. Only 23 years after it was first discovered, resistance to Penicillin was recorded in 1943, then, it was identified all over the world when methicillin-resistant *S.aureus* was reported in 1960. In the 1970s, carbapenem was produced, but pathogens quickly became resistant, after only 15 years. Notably, the resistance time is becoming shorter and shorter (20). In addition, the number of AMR-related deaths is estimated to become higher the overall deaths due to cholera, diarrhoea diseases, tetanus, diabetes, and measles combined and as well as higher than the number of people that die from cancers each year (21).

1.2. Impacts of AMR on human life and the environment

It has been widely understood that AMR does not only impact human health such as increased morbidity and mortality, but it also affects the economy, society, and environment. According to Mohsen Naghavi (2022), an estimated 25% (1.27 million) of people died from infections from antibiotic-resistant bacteria in 2019, compared to 4.95 million deaths associated with AMR. The leading pathogens that caused deaths in a human were *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (22). Treatment of these pathogens costs more than $2 billion each year (23), additionally, carbapenem-resistant *Acinetobacter baumannii is* estimated to be higher at $4.6 billion (24) and a cost of the total cost of all antibiotic treatment is estimated to be excess more than $1 trillion annually by 2050 worldwide (23). Moreover, patients infected with AMR bacteria needed

longer hospitalization, more intensive care, and are subject to fewer treatments with antibiotics (23). For instance, the average time of treatment for MRSA is 8 - 11 days longer in the hospital compared to methicillin-susceptible *Staphylococcus aureus* (25, 26), as well as third-generation cephalosporins resistant *E.coli* needed more than 5 days for treatment (27). For some hospital-acquired infections, the patient spent a 16 days in hospital with an estimated cost of an additional $ 35, (28).

While resistant pathogens were also found in the environment. *Acinetobacter baumannii* isolated from industrial effluents (29), *Escherichia coli,* and *Pseudomonas aeruginosa* from river water were identified to have a high resistance to antibiotics, related to heavy metals on their eco-niches (30), New Delhi Metallo-beta-lactamase 1 producing *Vibrio cholerae* NAG circulated in the river (31). These strains circulated in the environment and got transmitted to humans via a range of routes . The exchange of resistant genes between species in the environment and transmission to humans has been documented recently (32-34). As well as the transmission from animal to human has been reported (32).

1.3. Sources of AMR and Solutions

The imprudence and misuse and overuse of antibiotics are not the only reason for the rise in antibiotic resistance (34). The Centre for Disease Control and Prevention(CDC) estimated at least 30% of antibiotic prescriptions were unnecessary for outpatients and 20 - 50% of antibiotics administered was unnecessary or inappropriate at acute-care hospital in the USA (35, 36) and 30% of antibiotics were suboptimal or unnecessary in Western countries (37). In fact, 50% of prescription antibiotics for the first treatment started wrongly and without diagnostic proper of the pathogens (37).

The anthropogenic pollutants through industrialization, and urbanization is another reason that contributes to AMR health concerns (38). The bacterial evolutions, including mutation, against antibiotic resistance, is the way to adapt to environmental stress (39) and became less susceptible to antibiotics as well. In the

environment polluted with heavy metals, these sources were also another factor to induce bacteria to turn resistant to antibiotics (40-42). Figure 1.4 showed some mechanisms such as efflux system, detoxification of metal ions, bacteria adapted to heavy metals, then tolerance and increase resistance to antibiotics (figure 1.4).

Regarding the etiology of rising AMR, climate change has recently contributed to factors influencing AMR. Temperature is strongly associated with bacterial incidence and infections (43). It was estimated that a temperature increase of $1^{O}C$ could cause a rise in *Salmonella* cases by 5 - 10%, and the impact of this could be an additional 50,000 to 100,000 foodborne Salmonellosis illnesses and 27,000 annual hospitalizations in the USA(44). In addition, there is significant evidence that increasing temperature is associated with the rise of AMR (45-47). According to Derek R. MacFadden (2018), a temperature increase of $10^{O}C$ raised antibiotic-resistant of *Escherichia coli*, *Klebsiella pneumoniae,* and *Staphylococcus aureus* by 4.2%, 2.2%, and 2.7% respectively (48).

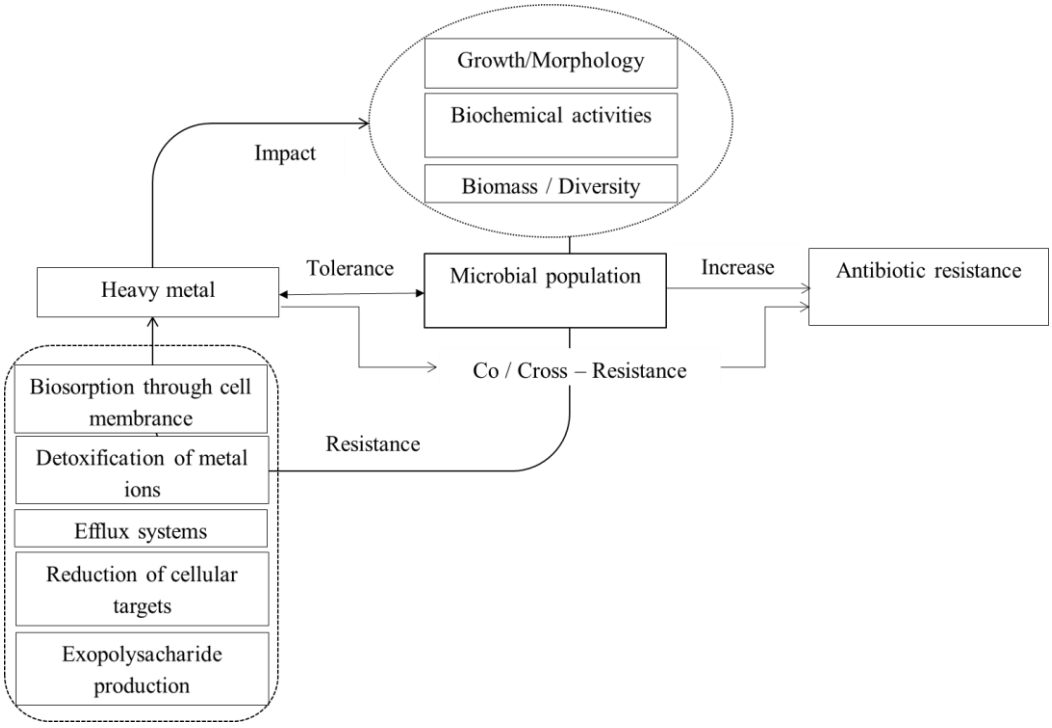

Figure 1.4: Relationship between heavy metal and antibiotic resistance. Source: Diep The Tai et al. (2022). The impact of heavy metals on bacterial tolerance of antibiotic resistance and growth in the aquatic environment of Vietnam. ***Infectious Diseases Research*** 2022;3(1):1. https://doi.org/10.53388/IDR20220201001

The increase in number of multi-resistant strains also highlights some of the challenges for effective treatment and stopping AMR from spreading globally. Nowadays, most solutions focused on developing new policies, including strengthening laboratory capacity, developing new guidelines at the national level, or adapting international level, training (49). Nevertheless, since 2017, only eight new antimicrobial agents have been approved and 50 antibiotics and 10 biologicals have been developed. Of these 50 antibiotics, 32 focus on WHO priority pathogens (50). The lack of new antibiotic treatments has led to many recent trials focusing on synergic effects from two or more different antibiotics (antibiotic - antibiotic), a combination of phage therapy and antibiotics or usage of herbal medicines with antibiotic's activity. Based on the synergistic effects, combining two or more antibiotics showed the amelioration of effective treatment (51, 52). Interestingly, the combination of phage therapy with antibiotics also improved the highly effective against multidrug-resistant strains in treatment (53-56). Notably, using phage therapy for the treatment of a wide range of infections started in 1920 (57) and recently renewed interest in combating antibiotic-resistant bacteria with many application models as well as an effective potential therapy to control multidrug resistance (58-62). Moreover, using antimicrobial peptides has also indicated better efficacy for the treatment of multidrug-resistant strains (63). Parallel to the changing treatment therapy, there has been significant innovation in new detection methods. Most of the new techniques for rapid detection of AMR techniques have focused on molecular biology, and real-time - detection (table 1.1).

Many issues need to be address on antimicrobial resistance such as time consumption, technologies and real-time detection, therefore many new technologies recently developed for rapid detection of a single bacterial strain, apply digital Loop-mediated isothermal amplification (LAMP) or use commercial test kit run with Malditof, Vitekk system to detect phenotypic antibiotic resistance. These methods were to detect pathogens in the blood, urine samples as well as on isolates. Despite most technologies trying to reduce the turnaround time, single strains isolated from many sources of samples are still used as the first step to

calculating time detection. This calculation, however, didn't count the time for isolating pathogens which also takes at least two to three days (table 1.1).

Table 1.1: Recently developed new methods for detection of AMR

Alternative methods	Specificity and Sensitivity, concordance, agreement	Very major errors (VME), major errors (ME), and minor errors (mE)	Samples	Species	Time	References
MALDI-TOFVITEKMS® system software version3.0 (bioM´erieux)	High concordance (0.87 – 0.99 for GN, p<0.001)	rAST, CA, VME, ME, and mE disclosed 97.7, 0.7, 0.5, and 1.1% for GN, 98.0, 0.5, 0.7,and 0.8% for GP, respectively	Positive blood culture	246 Gram-negative(GN) and 278 Gram-positive(GP) aerobes	4 – 6 h, compared to traditional methods	Dariane C. Pereira et al (2019). BioMed Research International
Automated VITEK 2® system	95% agreement for GNB and 96.1% for GPC	a minor error rate of 2.3%, a major error rate of 1.1%, and the very major error rate of 1.6%	Positive blood culture	37 were gram negative bacilli (GNB) and 28 were gram-positive cocci (GPC)	18 hours earlier than the standard method	Tamily Cristina Lemo et al. (2018). Rev Soc Bras Med Trop 51(2):215-218

Field-effect enzymatic detection (FEED - An immuno-detection platform.			Blood samples	Listeria innocua (ATCC 33090), *E.coli* 25922 or wilt type	204 min sample-to-result	Xuyang Shi et al. (2018), Scientific Reports | (2018) 8:3416
Digital LAMP quantification with SlipChip microfluidic devices, the phenotypic antibiotic susceptibility	51 correct calls (94.4% categorical agreement). Using the optimal threshold given by the ROC curve (1.11), 52 of 54 dAST calls matched the gold standard AST call (96.3% categorical agreement)	2 very major errors for 19 resistant samples (10.5%), and 1 major error for 35 susceptible samples (2.6%). 96.3% agreement with 1 very major error (5.3%) and 1 major error (2.9%)	clinical urine samples	*E.coli*, 54 clinical samples	15 min, less than 30 min	Nathan G. Schoepp et al (2017). Sci Transl Med.

The oCelloScope system – to analyse antimicrobial resistance by monitoring bacterial cell growth	compared to conventional susceptibility testing	96 % overall agreement, 3.6 % minor, no major, and 1.2 % very major errors	4 reference strains, nine clinical isolates, multi-drug-resistant isolates, and three positive blood cultures		95 % of the results being available within 180 min	M. Fredborg (2015). Eur J Clin Microbiol Infect Dis
Multiplexed, automated digital microscopy		96%		directly from clinical samples: positive blood culture or bronchoalveolar lavage fluid	5 hours	Christina Chantell (2015). Clinical Microbiology Newsletter
Rapid Automated Microscopy for Microbiological Surveillance of Ventilator-associated Pneumonia		100% sensitive and 97% specific			5 hours	Ivor S. Douglas (2015). Am J Respir Crit Care Med.

Single-cell morphological analysis (SCMA)		91.5%, 6.51% minor, 2.56% major, 1.49% major discrepancy	4 fours clinical, 189 clinical samples.		Less than 4 hours	Jungil Choi (2014)
Optical screening system (oCelloScope)			*E.coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, urine samples		6 min, 30 min with urine, and 3 hours for MIC (overnight culture - exponential phase)	Marlene Fredborg (2013). Journal of Clinical Microbiology.
Microplate-based surface area assay for rapid phenotypic antibiotic susceptibility testing. This assay					within 5 hours	Kelly Flentie (2018). 1Scientific REPORtS | (2019) 9:237 |DOI:10.1038/s41598-018-35916-0

measures bacterial concentrations by binding a universal small-molecule amplifier to bacterial surfaces						

Part 2 - Challenge of AMR and opportunities

2.1. Current assay to detect genotypic and phenotypic of AMR

Currently, most applied AMR diagnostic methods are based on genotypic and phenotypic assays, which are either performed manually or on automatic platforms. Conventional methods including disk diffusion tests, agar dilution, and broth dilution are manual. Some automated commercial kits such as the Vitek system(64), Phoenix system (65, 66), and Sensititre Aris 2X took longer than other systems (67) and have also been performed in microbiology laboratories. PCR-based methods, microarray, genome sequencing, and metagenomic (figure 2.1) have been applied as well as microfluidic technologies such as colourimetric assay, single cell or single-molecule (68), MALDI TOF mass spectrometry (69) and point of care which based on antibodies, aptamer, nucleic acid, and protein (70) have recently developed and provided many promising results. These methods, however, still have some disadvantages such as poor sensitivity, labour-intensive, expensive, cross-reactions, and no differentiation between viable or dead pathogens, even though most of the developed technologies were near the patient but still struggle to meet the standard criteria (37, 70, 71).

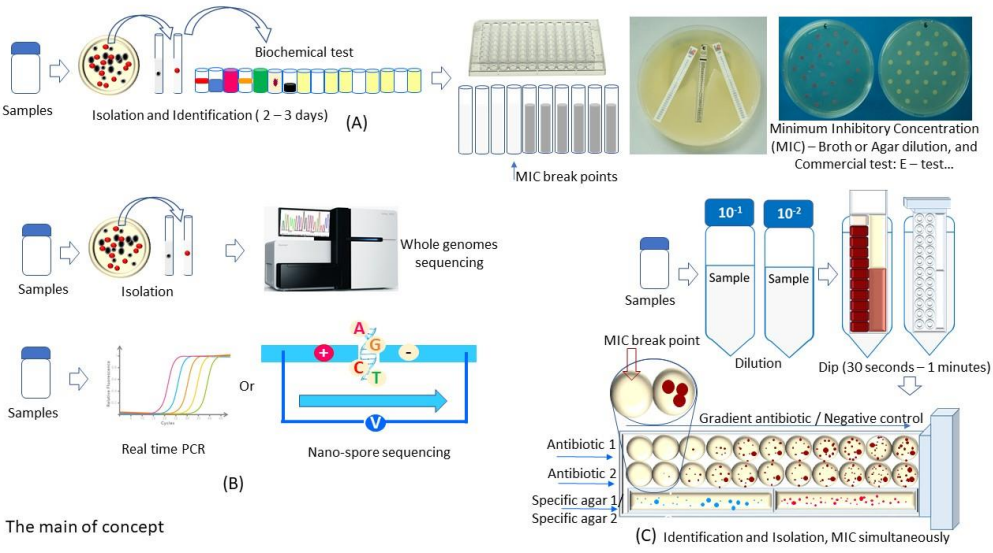

Figure 2.1: Current methods applied to the detection of AMR.(A). Traditional assays for performance of MIC. (B). Molecular assay. (C). Our developed assay.

With the need for rapid detection of infectious disease, many commercial tests kits based on phenotypic have been launched onto the market. Most of them are just used for the identification of pathogens for food and air quality control (table 2.1). Although these tests are easy to use, they are still time consuming , and some are expensive and have no detection of AMR.

Table 2.1: Commercial dip slide on the market

Order	Name of products	Company	Purpose	Price	Link
1	MCBTM2™ dip slides	Dip-slides	Test for aerobic bacteria, fungi, yeasts, and moulds	£11.96	https://dip-slides.com/kits/83-cosmetics-bacteria-yeasts-moulds-test-kit.html
2	Cosmetic dip slides, including GNAT MX2 incubator	Dip-slides	Test for a total count of bacteria and yeasts and moulds	£168.13	https://dip-slides.com/kits/83-cosmetics-bacteria-yeasts-moulds-test-kit.html
3	Dip-Slides™	Thermo Scientific	aerobic bacteria, yeast, and fungi with both side	?	https://www.thermofisher.com/order/catalog/product/R65205
4	Dip slide	Each Microbiology LTD	Bacterial/fungal in water	?	https://echamicrobiology.com/products/test-kits/dip-slides
5	Envirocheck ® Dip Slides	Merck	Detection of yeasts and moulds *Enterobacteriaceae* and total coliforms / *E. coli*	?	http://www.merckmillipore.com/GB/en/product/Envirocheck-Dip-Slides,MM_NF-C149015
6	Dip slide	DCS	dual-surface paddles for	£10.50	http://www.dcsproducts.co.uk/produ

			testing of Water Hygiene routine		cts/Dipslides-Box-of-10.html
7	Dipslides	*Legionella* Control International	Measures bacteria in liquids, on surfaces, and in air	?	https://legionellacontrol.com/legionella-products/dip-slides/

2.2. Raspberry Pi and 3D printed based methods

Beyond the need for real-time diagnostic, reduced turnaround time, and increase real-time detection, the combination many technologies into one test in order to increase the accuracy, convenience for users is an essential issue. With the need for the test's accuracy, a combination of digital computers, artificial intelligence, and portable communication technologies has offered an alternative way to tackle infectious diseases and AMR (72-74). Since its first release in 2012, the Raspberry Pi - a low-cost single-board computer has increasingly attracted researchers and is broadly applicable to image analysis when combined with a microscopy (75-77) and has increased widely in healthcare applications (figure 2.2). Just 9 years after its development, many projects are using Raspberry Pi in medical fields from temperature monitoring to image analysis have been exploited and are ever expanding (figure 2.2). Interestingly, the development of three-dimensional printing (3D print) has led to a new approach for customized labware in the microbiology laboratory or health care (78-80). The use of 3D printing technologies as a tool for the detection of bacterial antibiotic resistance, however, was lower than in healthcare applications. Since 2012, there were only a few scientific publications on healthcare applications, the applications have leapt forward in 9 years, nearly 70 times more publications in 2021 (figure 2.3). Although there are a small number of scientific publications compared to healthcare applications, the 3D printing technology has a great breakthrough in application for the detection of bacterial antibiotic resistance, the same as healthcare applications

did (figure 2.3). By using this technique, labware can be produced such as centrifuges (81), frame dip slides, culture disks for bacteria (78), antibody dispensers (82), flow cells for microscopy analysis (83), and a combination of 3D microscopy with Raspberry Pi computers (84, 85). By applying 3D printing, the researcher can structure and design microchannel networks to generate multidrug microfluidic concentration gradients (86) or microfluidic devices for the detection of AMR (87).

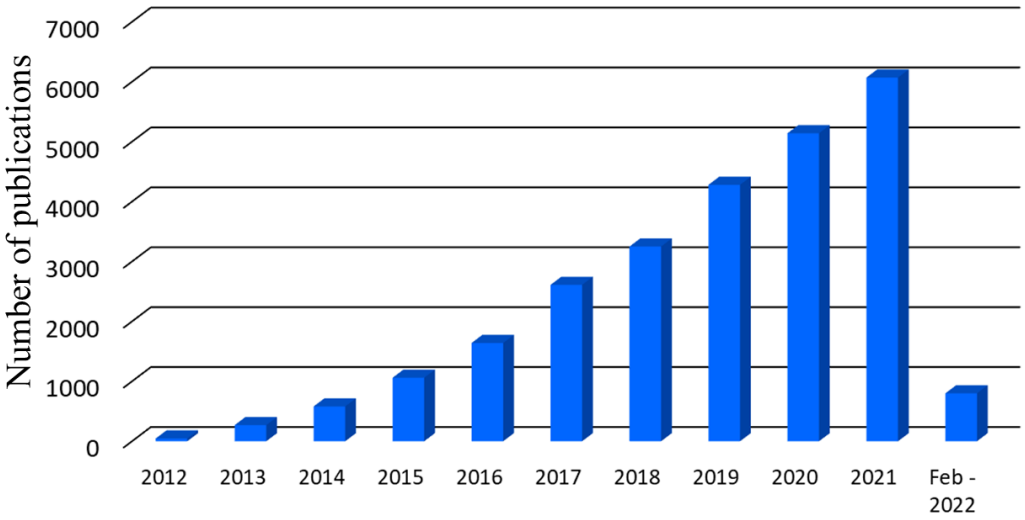

Figure 2.2: Increasing scientific publications on expanding Raspberry Pi for healthcare applications. Data was generated in full text by search on Google Scholar with the search term “Raspberry Pi for medical applications”. Data accessed until February 2022.

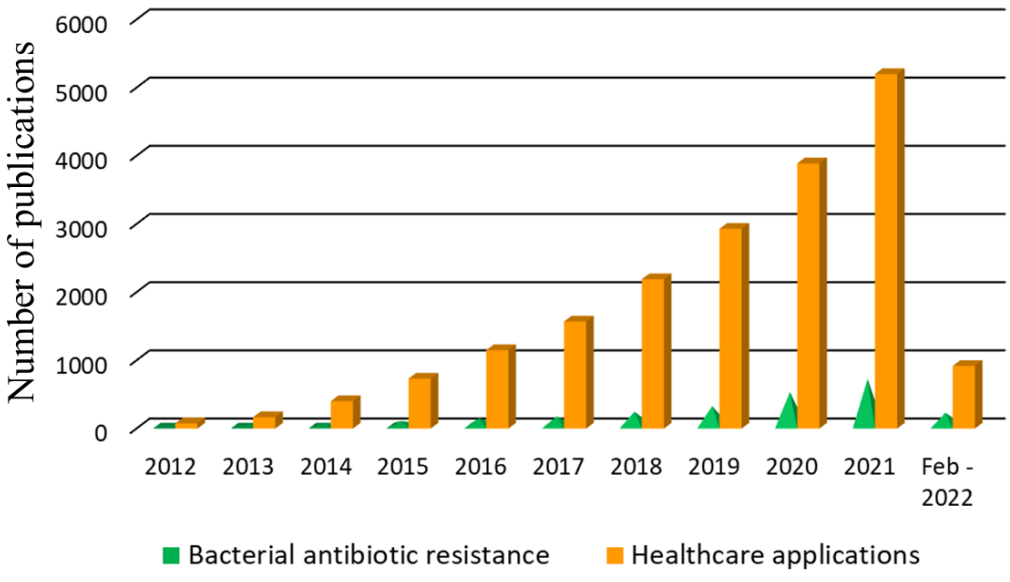

Figure 2.3: Comparison of the usage of 3D printing technologies in bacterial antibiotic resistance and healthcare application. Data generated in full text by search on Google Scholar with search term “3D printing technology for detection bacterial antibiotic resistance” vs “3D printing technology for healthcare applications”. Data accessed until February 2022.

2.3. Microfluidic technologies

Microfluidics has been critically studied for two decades focused on manipulating fluids in microstructure and recently expanded into the detection of AMR (88). Microfluidic-based techniques have provided single-cell analysis, molecular imprinted polymers (89), paper-based systems (90), and cartridges (91) to produce microfluidic devices to be used for point of care (POC) detection. These technologies have a wide range of applications such as quality inspection of milk and detection of contamination (92), biofilm formation and drug testing (93), drug discovery (94), multiplexed detection of foodborne pathogens (95) as well as detection of bacterial antibiotic resistance such as *Campylobacter* (96), *E.coli* (90, 97), *Klebsiella* (98, 99), *Mycobacterium tuberculosis* (94), *Staphylococcus* (99, 100) and *Pseudomonas aeruginosa* (101, 102). The advantages of microfluidics are speed - around 2 - 4 hours, being robustness, low cost, sensitive in analysis, and operating with a small sample volume (88, 94, 95, 101, 103). Despite these advantages, microfluidic technologies are still limited in their application in clinical settings. There are several reasons for not being broadly accepted and unpopular in clinical settings, for example fabrication, operational complexity, and requirement of sophisticated equipment and resources such as pumps, introducing running costs, and viscous forces (88, 103). In addition, trends of creating microfluidic devices were more popular than using microfluidic technologies only (figure 2.4).

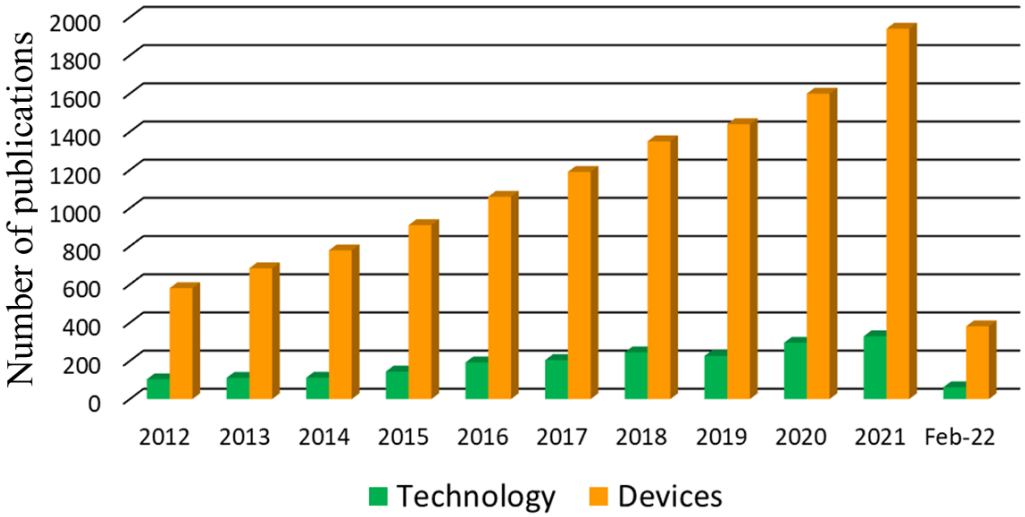

Figure 2.4: Trends of using Microfluidic technology. Data generated in full text by search on Google Scholar with search term “microfluidic technologies for detection of bacterial antibiotic resistance” vs “microfluidic devices for detection of bacterial antibiotic resistance”. Data accessed until February 2022.

In brief, phenotypic assays had larger developments than genotypic assays. The trends of new methods or alternative technologies were the ability to be portable, available price, real-time detection and easy to perform, with emphasis on small devices which can perform in various settings.

CHAPTER 2 - DESIGN MICROBIOLOGY LABWARE - 3D PRINT

Design microbiology labware - 3D print

This study elucidated the role of 3D technologies and their promising application in microbiology.

Full paper

Tai The Diep, Partha Pratim Ray, Alexander Daniel Edwards (2021). **Methods for rapid prototyping novel labware: using CAD and desktop 3D printing in the microbiology laboratory.** Lett Appl Microbiol 2022 Feb;74(2): 247 - 257. PMID: 34826147. **https://doi.org/10.1111/lam.13615**

The extent of Contribution to research:

In this work, I:

1. Designed some consumables for the microbiology laboratory
2. Designed the experiment
3. Performed the test
4. Collected and analysed the data
5. Drafted the manuscript

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ORIGINAL ARTICLE

Methods for rapid prototyping novel labware: using CAD and desktop 3D printing in the microbiology laboratory

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Significance and Impact of the Study: We present methods for designing and 3D printing microbiological labware offering alternatives to off-the-shelf consumables that allow low-cost rapid prototyping and customisation of microbial culture tools. We demonstrate customised 3D printed rapid prototype solid medium culture dishes and dip-slides, and modified inoculating loops and customisable replicating pins for plating bacteria. 3D printed labware can offer local production to avoid dependence on commercial suppliers, rapid customisation of existing designs, and rapid evaluation of entirely new tools. Using 3D printing to develop novel labware tailored to simplify routine work in the microbiology laboratory can save time and labour compared to relying on off-the-shelf mass-manufactured labware.

Keywords
3D printing, culture dish, dip slide, fused filament fabrication, loop, microbiology labware.

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Abstract

Although the microbiology laboratory paradigm has increasingly changed from manual to automated procedures, and from functional to molecular methods, traditional culture methods remain vital. Using inexpensive desktop fused filament fabrication 3D printing, we designed, produced and tested rapid prototypes of customised labware for microbial culture namely frames to make dip slides, inoculation loops, multi-pin replicators, and multi-well culture plates for solid medium. These customised components were used to plate out samples onto solid media in various formats, and we illustrate how they can be suitable for many microbiological methods such as minimum inhibitory concentration tests, or for directly detecting pathogens from mastitis samples, illustrating the flexibility of rapid-prototyped culture consumable parts for streamlining microbiological methods. We describe the methodology needed for microbiologists to develop their own novel and unique tools, or to fabricate and customise existing consumables. A workflow is presented for designing and 3D printing labware and quickly producing easy-to-sterilise and re-useable plastic parts of great utility in the microbiology laboratory.

Introduction

In the past, great steps forward in microbiology have been supported by methodological changes, often requiring custom labware, instruments or reagents produced in-house. The origins of modern microbiology lie with the development of tools such as the Petri dish, developed in the 1880s (Shama 2019), which allowed pure isolates to be cultured from colonies on solid media, a method that has barely changed in over a century (Lagier *et al.* 2015). Recently, the convenience of off-the-shelf products supplied by manufacturers has improved lab throughput, with the consequence that microbiology labs can become dependent on supply of conventional components from commercial suppliers. Innovative analytical techniques remain critical to human health and wealth, and underlying most analytical methods and microbial research lies microbiological labware. Rapid prototyping of plastic objects has never been simpler or more accessible, and the potential for 3D printing in research is expanding and

expected to lead to a new approach where microbiology labs can adapt and customise labware for individual applications. Fused filament fabrication (FFF) 3D printers retailing for under £200 are reliable, easy to use, and widely available across the world. While more advanced rapid prototyping methods are becoming more common, this simple approach produces a wide range of different shaped plastic objects, suitable for many laboratory activities. The material cost of parts runs around £20 per kg, plus labour cost associated with operating the printers. Custom labware offers opportunities to rapidly innovate to solve common problems in microbiology laboratories.

In-house designed and 3D printed labware can offer at least three benefits: Firstly, 3D printing offers the chance to locally make labware that is identical to standard consumables, but without being dependent on commercial suppliers/distributors and thus at lower cost, available faster, or avoiding delivery delays (for example during supply chain disruption). Secondly, modified versions of conventional/commercial products can be designed and 3D printed that are customised to individual needs (for example, different shape agar plates to conventional Petri dishes). Finally, entirely new tools can be created and evaluated rapidly, allowing rapid iterative development of new methods. Here, we focus on rapidly creating modified versions of conventional culture tools. For example, whilst round Petri dishes are suitable for a wide range of methods, they are often inconvenient for example not being compatible with microplate well grids and multichannel pipetting. Alternative square dishes are less widely available and still don't correspond to the standard 12 × 8 grid and 9 mm pitch of 96-well microwell plates. Dishes with multiple compartments are less common, partly because different assays need different sized compartments, but replica plating samples onto multiple agar types is a common procedure, requiring large stacks of Petri dishes and large volumes of solid media. Whilst standardisation is extremely helpful for automation and instrument development (e.g. microplate readers), microwell plates themselves are only available in a limited range of configurations of well sizes, numbers and arrangements. They are most commonly produced for eukaryotic tissue culture or molecular biology, rather than being configured specifically for microbiology methods such as culture and functional/phenotypic assays (Maia Chagas *et al.* 2017). As 3D printing allows rapid production of any shape or size of culture dish to be customised, it can support high throughput testing in multi-well solid agar plates configured for specific microbiology methods.

To create new microbiological labware for research and diagnostics fabricated commercially by typical mass-manufacture methods such as injection moulding or thermoforming, researchers would need close support from vendors, manufactures and suppliers. The cost for final mass-produced components and prototypes will be expensive for unique or unusual objects and designs because of the high capital cost of tooling plus cost of experts to redesign, and researchers may need to buy in bulk without being able to obtain one piece for a quick evaluation (Neches *et al.* 2016).

Outside the distribution network of the major suppliers of microbiology consumables even conventional labware is not always accessible. In many parts of the world, it can take four or more weeks to receive materials for experiments, further limiting the opportunity for rapid testing and innovation. Purchasing power parity distorts the price of scientific research materials, combining with high shipping costs, and significantly limiting the availability of microbiology labware and materials. This can delay uptake of the latest methodology, and restrict microbiology labs in low resource areas to older methods (e.g. round Petri dishes). Even in well-resourced laboratories with rapid access to commercial labware, supply-chain disruption (e.g. shipping blockage) and depletion of critical components (e.g. during pandemic) can be overcome if individual labs can produce their own in-house labware. Yet the need for global analytical microbiology both for local public health and for coordinated surveillance, especially for infectious agents and antimicrobial resistance genes that can spread rapidly across the world, continues to drive a need for more, better, analytical microbiology in all regions, especially those with lower resources for accessing innovative lab tools.

Another global microbiology pressure is that current gold-standard culture methods are often time-consuming to simultaneously perform on many samples, making it challenging to address outbreaks or for surveillance. They remain labour intensive, placing high costs to microbiology laboratories of vital importance for public health. For example, at least four different media might be needed to identify critical types of pathogen in each sample. One Petri dish contains 20 ml of each media type, therefore, 2000 ml of each media and a total of 8000 ml media will be prepared and sterilized to process 100 samples. It is estimated that 400 Petri dishes will be disposed of and take more than 200 min to complete just one step of the bacterial identification procedure. This could be streamlined if round plastic Petri dishes were replaced with smaller dishes that combine multiple media, to significantly reduce the volume of media and associated labour. Similarly, inoculation loops, frequently used for subculture or streaking out colonies, remain unchanged for many decades. Single-use plastic loops are very similar in design to metal wire loops, yet plastic permits many different configurations. These could be customized in size to fit with other common lab formats, for example by

combining 96-well plates to process many samples simultaneously, without needing expensive multichannel pipettes for plating. Likewise, dip slides have in some applications replaced loops and Petri dishes for plating, saving labour and time of staff in the laboratory, but these are only available from a few suppliers in simple configurations with only one agar type per dip-slide.

The accessibility of 3D printing driven by the rise of Rep-Rap FFF rapid prototyping (Gross *et al.* 2014) now offers an opportunity to rapidly customise plastic microbiology labware. Rapid prototyping methods including additive manufacturing and three-dimensional printing have been widely applied for industry as an innovation tool, and these methods have increasingly spread into life science, medical and healthcare research. This was first introduced in 1986 by Charles Hull as a manufacturing tool, by designing bespoke objects created from 3D design software, then fabricating solid objects through layer-by-layer printing of stl files (Sharafeldin *et al.* 2018). Current biomedical applications include medicines, dentistry, pharmaceutical development, bioengineering, and medical devices (Awad *et al.* 2018; Sharafeldin *et al.* 2018; Gonzalez-Henriquez *et al.* 2019; Culmone *et al.* 2020). With such benefits as flexible design, cost-saving and eco-friendly environmental material, 3D printing allows prototyping and manufacture of many research tools. Biocompatible parts similar to biological tissue such as bones, heart valve have also been explored (Culmone *et al.* 2020). In the microbiology field, examples have been published including a 3D printed motility assay device (Neches *et al.* 2016), and digital microscopes for the microbiology laboratory (Neches *et al.* 2016; Maia Chagas *et al.* 2017; Del Rosario *et al.* 2021), however, we do not yet see widespread uptake.

We believe many microbiology researchers and analytical laboratories can benefit from in-house rapid prototyped labware, by adopting CAD and desktop 3D printing methods. We propose that the latest desktop 3D printers and open-source CAD software are now accessible enough for non-engineers to adopt into their labs. In this paper, we describe practical methodology for introducing-free open-source CAD plus low-cost desktop 3D printing into a microbiology research laboratory, to rapidly prototype and manufacture custom microbiology labware. We illustrate the power of 3D printing to replace Petri dishes and pipettes by designing, fabricating, and testing novel customised frames to create multi-agar dip slides, customised inoculation loops, bespoke multi-channel dishes for replica plating onto a panel of agar types, and replicator pins in different configurations. For these designs, we maintain compatibility with 96-well microplates to simplify processing of multiple samples, and at the same time interface with current labware standards. For these examples, we outline the methodology of design, 3D printing by FFF rapid prototyping, and present qualitative validation in the microbiology laboratory.

Results and discussion

Rapid prototyping methods for in-house design and fabrication of microbiology labware

Through FFF 3D printing, custom labware can be designed or redesigned to replace commercial consumables or to create new tools. The process of creating a novel consumable needs several steps from design to manufacture and testing (Fig. 1). We highlight key practical considerations that a microbiology laboratory would need to consider when starting to 3D print labware (Table 1). An increasing number of repositories host open-source designs freely available to download and 3D

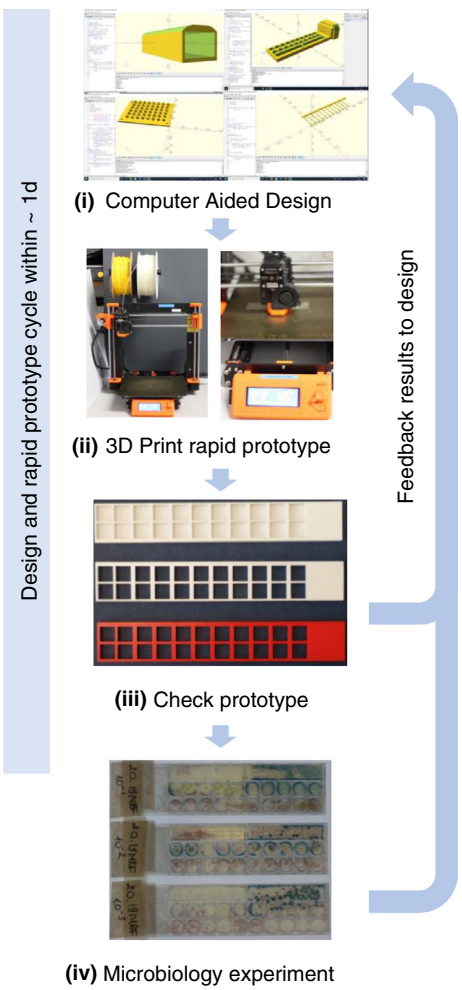

Figure 1 Method and workflow for rapidly prototyping custom microbiology consumables using CAD and desktop FFF 3D printing.

Table 1 Key considerations for rapid prototyping and 3D printing microbiology labware

Topic	Barrier	Cost	Evaluated in this manuscript	Alternative	Conclusion
CAD software	Can you access CAD package?	Free Open-Source and free licenses to proprietary CAD packages available	OpenSCAD	FreeCAD or Fusion360. Some advanced software can be licensed but many academic institutions have license to more advanced CAD packages for research and teaching	CAD software now widely available, both free open-source and commercial products
CAD skills	Can a microbiology researcher design 3D parts for rapid prototype testing?	Many online training resources available free of charge	New designs created and refined by doctoral research student	Can contract engineer to design parts. Can collaborate with engineering department	Basic skills for simple models can be gained in a few days. More complex designs need more expertise
3D model design	Has what you need already been designed?	Growing libraries of open-source 3D models available free of charge	In-house design by microbiology researcher	Can download many 3D designs; can collaborate with engineer or use consultancy	Design time for in-house CAD fits within scope of doctoral research
3D printing hardware	Can you access a 3D printer?	Basic desktop 3D printer costs significantly less than £200	Prusa i3 MK3 (£700 from Prusa Research, Prague in 2019) and Creality Ender 3 (£190 from Farnell, Leeds UK in 2020)	Many 3D print services offer fast prototyping. Makerspaces and universities have shared 3D printing facilities	Capital cost of basic 3D printers no longer a significant barrier
3D printing skills	Can you operate a desktop 3D printer	Many online training resources	Printed in-house by doctoral research student	3D printing facilities and services offer technical support for printing	Sufficient time is required for researcher to operate and troubleshoot 3D printer
3D printing materials	Are 3D printed parts compatible with microbiology experiments	£20 per Kg for poly-lactic acid (PLA) in 2020	Poly-lactic acid (PLA) from multiple local suppliers effective for the applications we tested	Polyethylene tetraphtalate (PET) and acrylonitrile butadiene styrene ABS (multiple suppliers) offer increased heat resistance	Cost of 3D printing consumables lower than conventional microbiology lab consumables

print, so in-house design is not always necessary although these are not always straightforward to use (Alcock *et al.* 2016). For in-house development, we use OpenSCAD open-source computer-aided design (CAD) software (available from www.openscad.org) because it is freely available and the open-source model permits anyone else to open and/or edit our design files. However, proprietary CAD software is also available, with many educational establishments having access to more advanced CAD packages for research and teaching. Using OpenSCAD software, we can create and edit a bespoke model (step 1) that can be openly shared for others to edit. This software is parametric, allowing critical dimensions to be defined and rapidly changed, for example size or number of wells in an agar dish. A 3D object file is then exported in 'Standard Triangle Language' or 'Standard Tessellation Language' (.slt) file format which then needs to be 'sliced' into many layers to be 3D printed using open-source software configured for the desktop 3D printer. The sliced file is then transferred to the desktop 3D printer (Fig. 1, step 2). In step 2, all parameters for printing including temperature, *z* axis, layer resolutions, and speed, can be modified if desired, or generic settings used that are supplied with most 3D printers. Printing settings need optimisation for properties such as mechanical strength and stiffness and for microbiology labware, these often need tuning to ensure parts contain liquid without leaking. Many printing parameters will be selected to match the 3D printer material and printer used locally, so we cannot provide general rules for 3D printing microbiology labware, however, we have successfully used generic settings supplied with the printer for PLA. We almost exclusively print microbiology research labware using polylactic acids (PLA) plastic, despite some limitations of its material properties. Although many other options are available such as polyethylene tetraphtlatate (PET) or acrylonitrile butadiene styrene (ABS) which are more stable at higher temperatures than PLA, we have never found significant advantages, and PLA is simpler to print using inexpensive 3D printers. To print smaller parts

(e.g. <10 × 10 × 1 cm objects) producing the prototype parts just takes a few hours; 1 day or overnight is needed either for larger objects or to print a large batch of smaller objects.

Sterilisation of 3D printed microbiology labware

Sterility is a crucial requirement in microbiological methodology and this is also the most important question for any protocol or material to be used in a microbiology laboratory (Neches *et al.* 2016). PLA has a low melting temperature to simplify 3D printing, and softens in boiling water, but surprisingly we found it possible to autoclave PLA labware parts, if some care is taken during sterilisation. We found that autoclave could only be used for PLA parts when individual pieces were carefully wrapped with aluminium foil and placed on a flat surface during autoclaving, and allowed to fully cool before removal. Although some minor changes in shape occurred, for simple parts the functional shape was retained and overall dimensions remained within 2% of original (e.g. an 100 mm long dip-slide frame only shrunk by 1 mm after autoclaving). However, if parts were placed together in a pot for autoclaving, their shape changed after autoclaving, becoming unusable (Fig. 2). The amount of distortion after autoclaving depended greatly on the shape and size of the parts; so every design must be checked for compatibility with autoclaving. We found ABS parts less sensitive to deforming as expected from its higher melting temperature, so alternative 3D printer materials such as PET and ABS may therefore be more tolerant of microbiology autoclaves. However, we found the convenience and speed of printing PLA parts outweighed this improved heat resistance.

The simplest option was sterilisation with 70% alcohol, followed by drying, which we found very effective and reliable to sterilise PLA labware with no loss of function or damage to this plastic. PLA parts couldn't be used for microbiology culture directly after printing, because although the melt-processing at above 200°C during printing will sterilise parts (Neches *et al.* 2016), our laboratory 3D printer is not maintained in an aseptic environment and so prototype parts were unsurprisingly not sterile (Fig. 2). Installing the 3D printer into an aseptic cabinet would avoid this, but would dramatically increase the cost and space requirements, as laminar flow HEPA-sterilised workstations are larger and more expensive than the 3D printer within; furthermore, the airflow would be likely to affect the printing by changing the airflow and cooling speed of polymer after extrusion—3D printers are known to be sensitive to drafts. Using an enclosed 3D printer might also be beneficial for part sterility, although these can be more expensive than the cheapest desktop 3D printers without enclosure.

Some changes to materials properties may occur after sterilisation either with autoclaving or 70% ethanol treatment, however, this class of polymer can be degraded by autoclaving (Rozema *et al.* 1991) we found that all parts retained adequate mechanical strength. Different materials (even the same filament type from different supplier) and other printing parameters also alter mechanical properties of 3D printed parts, and every lab must therefore check the suitability of parts in-house. Furthermore, potential interference with culture container material with microbiological experiments must be checked in-house, for example possible leaching of plastic additives that might interfere with antibiotic susceptibility or growth assays, in common with conventional labware. We explored if some 3D printed labware parts could be re-used if needed as an alternative to single-use and disposal. While PLA is often labelled as a biodegradable polymer, re-use could also contribute to solving the overuse of single-use plastic labware recently which contributes to pollution globally (Chen *et al.* 2021). We found that careful washing followed by re-sterilisation using 70% ethanol was adequate to permit re-use, and simple PLA parts remained functional for containing agar medium when used at least five times before deteriorating. Beyond five wash—ethanol sterilisation—and reuse cycles, the dip-slide frames became too brittle to use.

Sterilisation of 3D printed parts has been explored elsewhere, not only with reference to microbiology (Neches *et al.* 2016) but also in bioscience research and medical device fields. For example the effect of autoclaving on material properties of 3D printed medical devices and surgical implants has been extensively studied (Boursier *et al.* 2018; Aguado-Maestro *et al.* 2021; Pérez Davila *et al.* 2021). Likewise, 3D bioprinters have been established for cell culture where sterility is vital (Kahl *et al.* 2019).

Preparation of custom solid medium configurations in dip slide frames for direct sample plating onto multiple media

Direct detection of pathogens from samples is a major concern for the microbiology laboratory, often tackled using culture methods including an array of identification media that select certain species and colour colonies. Conventional labware requires stacks of Petri dishes, increasing labour and taking up space in refrigerators and incubators. We designed 'dip slide frames' containing two different solid agars in multiple configurable wells (Fig. 3a). Long rectangles provided larger areas of agar suitable for colony identification, and we added two rows of circular wells to permit use for comparing growth on

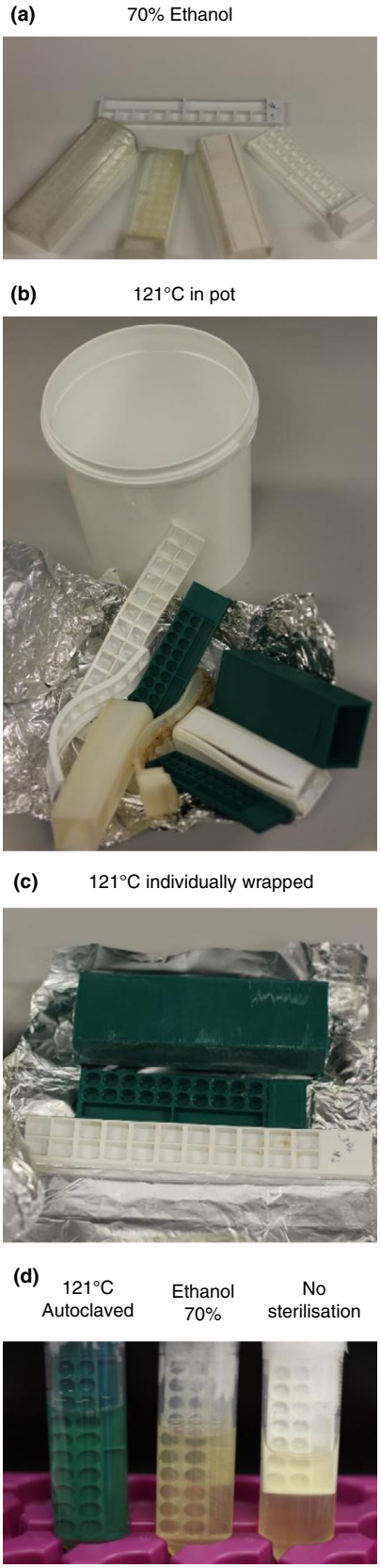

Figure 2 Sterilisation of 3D printed PLA labware. (a) Illustrates set of parts unchanged by sterilisation with 70% ethanol. (b) When bulk autoclaved in a pot, the heat-softened parts were badly distorted. (c) However, if carefully positioned flat on a tray in individual foil wrappers, minimal distortion was seen and parts could be used after autoclaving in spite of softening during heating. (d) Parts were either added to broth medium either without sterilisation or after autoclaving or 70% ethanol sterilisation; after overnight incubation in broth, the culture medium remained clear only following sterilisation, indicating printed parts were not sterile directly after fabrication.

smaller areas of other solid agars—for example with added serial dilutions of antibiotics to permit direct determination of minimum inhibitory concentration (MIC; agar dilution method, manuscript in preparation). To illustrate and evaluate the design concept, we used just two Chromoagar types (gram+ and gram−) in the whole frame and cultured a mixture of *Escherichia coli* and *Klebsiella pneumoniae* illustrating the different colony colours on the media—pink for *E. coli*, and blue for *K. pneumoniae*. These frames are used in the same way as commercial dip-slides by just dipping directly into liquid samples and overnight incubation at 37°C. These 3D printed frames allowed us to rapidly test the feasibility of including multiple solid media in a single dip-slide (Fig. 3b); using multiple sizes to determine the smallest needed to detect the growth of particular targets.

Using these prototypes, we discovered that the light sources and exact material of PLA used to print the parts were crucial factors to record and interpret the results (Fig. 3c). In our data, natural transparent PLA was the best choice material to get a clear image recording colony growth, compared to other colours or materials. For example, it was possible to see the colonies by eye or digital photograph on the surface of solid agar under room lighting with blue or white PLA material. However, with white light illumination under the parts, the coloured and white PLA were opaque preventing illumination, making it hard to observe colonies on the lightbox. In contrast, the natural PLA was transparent allowing white backlight to illuminate the bacterial colonies, and giving good colour record of colony type, helpful for the identification of bacteria on chromogenic identification media. We illustrated this with three dilutions of a mastitis milk sample (Fig. 3d). Having established this design concept is feasible, we are working further to explore the performance of this design for rapid direct testing (manuscript in preparation).

Preparation of custom solid medium plates and plating loops in diverse formats

Processing multiple samples are simplified by microwell plates, yet the size of common solid media culture dishes

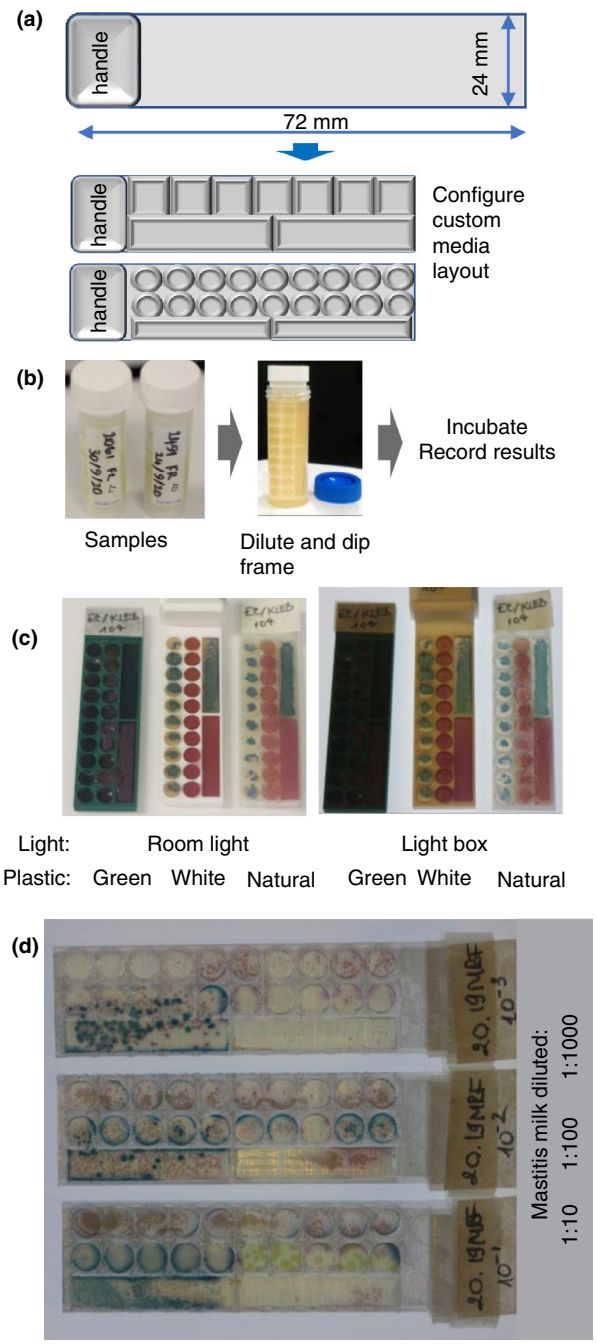

Figure 3 Customisable dip-slides for direct sample plating onto multiple agar types. (a) Customisation options. (b) Operation of custom dip-slides for sample plating. (c) Frames printed with the indicated PLA colours were illuminated from above or below and imaged to show how natural PLA with backlight makes colonies most clearly visible. (d) Example of mastitis milk sample plated onto custom dip-slides.

does not match conventional 9 mm pitch microwell plates. 3D printed cultureware can be configured to suit microwell plates, and forto specific test procedures. To illustrate this, we designed customized multi-well agar plates and loops (Fig. 4a) allowing us to streak out panels of samples from standard microwell plates onto solid medium. A 3D printed row of loops (Fig. 4b) with 9 mm matching 96-well plate format simplified plating by allowing an entire row or column to be inoculated onto agar in one go (Fig. 4c). We found that modifying the loop diameter (Fig. 4d) allowed us to change the volumes of the sample inoculated onto agar, ranging from ~10 µl down to ~1 µl, clearly demonstrated by the similar numbers of colonies deposited by loop *vs* micropipette (Fig. 4e). The 3D printed loops behaved similarly to commercial disposable plastic inoculation loops—but—it is worth noting that careful technique is still required as with any loop inoculation method to deposit equal quantities; loops are not a direct substitute for precise volume dispensed by pipettes. On the five-column multi-media plate, Baird Parker, Chromoagar for gram-negative and gram-positive, Mackonkey, and Mueller Hinton (MH) agar were prepared that would permit culture and identification of a wide range of species such as *Staphylococcus aureus, K. penumoniae, E. coli* and *Pseudomonas aeruginosa*. Each medium type spreads into 10 compartments but with boundaries separating the individual plated samples, reducing the chance of cross-contamination. We combined this multi-media plate with multi-well inoculation loops to plate out a set of reference strains in parallel (Fig. 4c). Using the multi-well plate reduced the volume of media significantly compared to conventional Petri dishes. The multi-well plate reduced the common problem of cross-contamination or fusion of droplets when plating multiple samples from 96-well plates onto agar in a Petri dish. Using smaller loop size could also reduce the volume of liquid deposited, further reducing the risk of cross-contamination. 3D printed labware is ideal for applications where droplet fusion or cross-contamination is a problem, as designs are not limited to standard 9 mm pitch between microwells in wells for commercial products, so wider spacing is possible. Diluting wells, inoculation loops, and agar dishes be designed together to meet custom needs.

Customised pin replicator allows personalised configuration for plating onto antibiotic plates to measure minimum inhibitory concentration

While commercial Petri dishes and microplates may be as cheap as 3D printed versions, other labware and tools may be hard to access with a high initial purchase cost. A range of steel pin replicators are available but can cost £500 or more to purchase, which may not be justified for a small experiment or evaluation. Furthermore, only a few configurations are available. 3D printing allows a

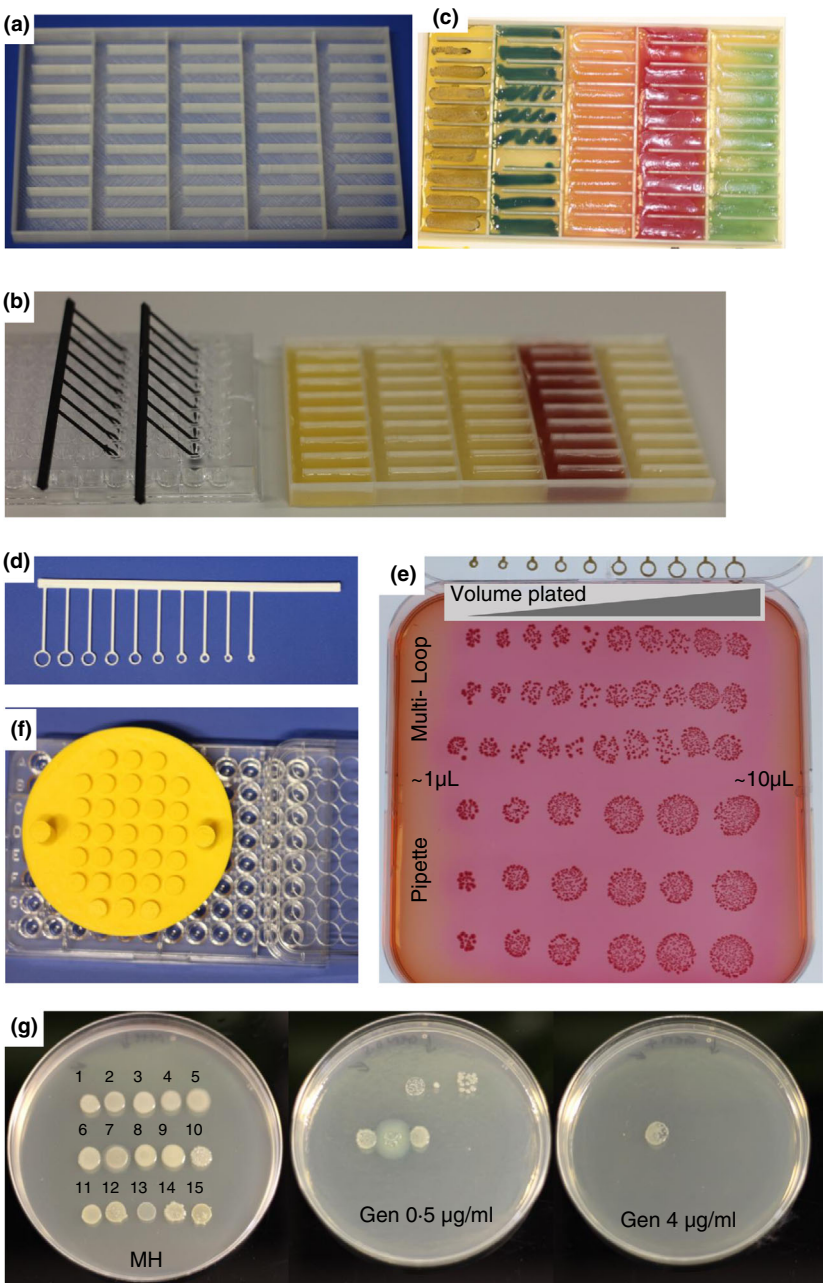

Figure 4 Fully configurable multi-chamber plates, inoculation loops, and pin replicator allows customised plating onto custom shapes of solid media. (a) Multi-chamber dishes for agar allow multiple samples to be separated but plated onto the same agar, poured into each connected column. (b) 96-well plate compatible loops were designed, printed and used to plate panels of samples onto five agar types in the custom plates. (c) Growth of bacteria streaked onto five different solid media. (d) Loop size can be customised allowing different volumes to be plated. (e) Illustration of loops for plating a series of culture volumes using variable-diameter loops (top 3 rows of spots) *vs* micropipette spots (lower 3 rows, comprising 1, 2, 4, 6, 8 and 10 µl culture medium respectively from left to right). (f) Illustration of pin replicator designed for 96-well plate and round Petri dish. (g) Example of 15 bacterial strains replica plated on Mueller Hinton (MH) *vs* differing concentrations of gentamicin in conventional Petri dishes, to determine MIC by agar dilution.

laboratory to evaluate the usefulness of a device such as a pin replicator prior to investing in commercial hardware, as well as rapid customisation. Through open-source design with open-source software and FFF fabrication, two formats of pin replicators were rapid prototyped with 31 pins (designed to fit conventional round Petri dishes

when square dishes are unavailable) and with 48 pins (for custom 3D printed square dishes), allowing replication of bacteria from 96-well microplates with a standard 9-mm pitch. We used these for agar dilution on conventional Petri dishes, and for replicating onto 3D printed customised multi-well plates. Fifteen isolates including multiple mastitis sample isolates plus reference strains were replica plated from colonies prepared in a microwell plate, and found to have significant resistance to cefoxitin and streptomycin, with variable sensitivity to gentamicin (Fig. 4g and data not shown). These tools offer an alternative to plating using micropipette or multichannel pipettor, or to commercially available pin replicators that are only available at a high price in a few limited configurations. Custom configurations of pin replicators may be helpful to fit with different multiwell plates/Petri dishes or to replica plate spots of different size or shapes of inoculum onto solid media.

Fused filament fabrication technology can become an essential tool to facilitate innovation in the microbiology laboratory. Whether producing in-house versions of existing labware to avoid supply problems, customising standard labware for specific methods, or developing entirely new components, our method guide and findings, can help researchers create or replace many types of labware without waiting for vendors to innovate or for delivery of supplies, as well as quickly iterating any parameters for experiments. With recent developments of FFF technology and inexpensive desktop 3D printers, it has become possible for researchers to design whatever they want and test in few hours. This will save time, ease research budgets, and ultimately reduce turnaround time to improve the quality of microbiology. Although we provide examples for plating and replication of bacterial samples to determine MIC on agar, plus dip-slides and plates for multichannel agar media, many different labware components can be readily designed and tested. Other equipment in the microbiology laboratory has also been designed and 3D printed including digital microscopy (Sharkey *et al.* 2016), centrifuge (Byagathvalli *et al.* 2019) and micropipettes (Brennan *et al.* 2018). The open publication of these designs will support a new wave of microbiology innovation across the globe.

Material and methods

Design files for loop and frame dip slide, multi-channel rectangle dish and pin replicator

All openscad and stl file of the labware in this study are available to download as electronic supporting information and details of these files are available in Table S1. All original OpenSCAD design files are available under an open-source hardware license, for open re-use and modification, and as well as downloading these from ESI they can all be accessed via a GitLab repository (https://gitlab.com/AlEdwards/various-lab-designs) and a zenodo repository (https://doi.org/10.5281/zenodo.5724098) with DOI: 10.5281/zenodo.5724098.

Bacterial strains

Escherichia coli ATCC 25922, *P. aeruginosa* ATCC 10145 and *K. pneumoniae* ATCC 13883 and *S. aureus* NCTC 8355 were used for replication test and streak on agar media in this study. These strains were inoculated overnight at 37°C on MH agar (Thermofisher, Basingstoke, UK). Alongside reference strains, 13 isolates from mastitis using Chromagar was also used. Replica plated strains numbered in Fig. 4g were as follows: spot numbers 1, 2, 5, 6 *E. coli* isolates plus 8 ATCC 25922 reference; 3, 4 *K. pneumoniae* isolates and 9 ATCC 13883 reference; 7 *P. aeruginosa* ATCC 10145; 11–15 *S. aureus* isolates plus 15 NCTC 8355 reference.

Sterility testing

After printing, replicate labware samples were be divided into three parts: one set was autoclaved by wrapped with aluminium paper either individually or put together in a pot, the second set were 70% alcohol sterilised and last set unsterilised. After sterilisation, all pieces were put into 10 ml LB media in a 50 ml tube and incubated at 37°C to check for contamination. Successful sterilisation was achieved when no growth of bacteria was detected, with clear liquid media indicating passing the sterility test. In this 3D printing methodology study, sterility tests were performed overnight, to confirm parts were suitable for overnight culture of rapidly growing strains. For experiments requiring longer incubation, a longer sterility test would be more appropriate, to exclude the possibility of contamination with slower-growing organisms.

Direct detection pathogens from milk samples

Mastitis milk samples were collected by the University of Reading farm and transported to laboratory to perform test. These were diluted at three concentration (10^{-1} to 10^{-3}) for testing. Dip slide frames, designed with 10 round or squared shape was 72 mm length and 24 mm wide and divided into two parts: one set of small chambers suitable for determining MIC (Taga and Bassler 2003) and the long part for identification of pathogens. Next, selective media was filled up into wells formed by the frame, and the dip-slides were then used by dipping directly into milk samples at the three dilutions and

overnight incubation at 37°C. Dip-slides were imaged by digital photography either with ambient room light or on a USB white light tracing box (Amazon, UK).

Plating bacterial samples with loops and multi-well plate

The Baird Parker agar (BP), MacConkey's agar (MC), Selective Chromoagar for gram negative and gram positive, Mueller Hinton agar were prepared according to manufacturer instructions then poured into multi-channel rectangle dish with 11 ml of media for each row. Then, sterile loops were applied to take the bacterial solution from 96-well plate to streak or drop bacteria into media, and incubated overnight at 37°C.

Replica plating for minimum inhibitory concentration agar dilution

The protocol to perform MIC-agar dilution followed CLSI guidelines. Strains isolated from mastitis samples and reference strains were prepared at standard inoculum density in 96-well plates. Cefoxitin, gentamycin, and streptomycin (Sigma, Gillingham, UK) were prepared as stock concentration, then diluted into Mueller Hinton agar and filled into round Petri dishes. The pin replicators were used to transfer the inoculum from 96-well plates onto the surface of Mueller Hinton agar, followed by incubation at 37°C overnight.

Acknowledgements

We would like to thank Barney Jones for providing spare mastitis milk samples from the University of Reading farm.

Conflicts of Interest

None.

Authors contribution

Conceptualisation—DTT and AE. Methodology and design—DTT and AE. Investigation—DTT. Writing—original draft—DTT. Writing—review and editing—PR and AE.

Data availability statement

All stl and openscad file of the labware in this study was uploaded to Mendeley data and Zenodo (https://doi.org/10.5281/zenodo.5724098) and the data that supports the findings of this study are available in the supplementary material of this article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary overview of design files and stl file downloads.

CHAPTER 3 - FRAME DIP SLIDE - PHENOTYPIC AMR

Frame dip slide - phenotypic AMR

This study showed evidence how the effectiveness of 3D frames and digital cameras in the early detection of antimicrobial resistance

Conference Abstract (Russian - Vietnamese Scientific and Practical Conference - Moscow 2019)

Tai The Diep (2019). Antimicrobial resistance on isolates from the human, environment in Southern region, Vietnam. **Oral Presentation.**

Full paper

Tai The Diep, Samuel Bizley, Alexander Daniel Edwards (2022). **3D-Printed Dip Slides Miniaturize Bacterial Identification and Antibiotic Susceptibility Tests Allowing Direct Mastitis Sample Analysis.** Micromachines 2022, 13, 941. **https://doi.org/10.3390/mi13060941**

The extent of Contribution to research:

In this work, I:

1. Designed the experiments and prototype of the frame.
2. Performed the test
3. Collected and analysed the data
4. Drafted the manuscript

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MDPI

Article

3D-Printed Dip Slides Miniaturize Bacterial Identification and Antibiotic Susceptibility Tests Allowing Direct Mastitis Sample Analysis

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Abstract: The early detection of antimicrobial resistance remains an essential step in the selection and optimization of antibiotic treatments. Phenotypic antibiotic susceptibility testing including the measurement of minimum inhibitory concentration (MIC) remains critical for surveillance and diagnostic testing. Limitations to current testing methods include bulky labware and laborious methods. Furthermore, the requirement of a single strain of bacteria to be isolated from samples prior to antibiotic susceptibility testing delays results. The mixture of bacteria present in a sample may also have an altered resistance profile to the individual strains, and so measuring the susceptibility of the mixtures of organisms found in some samples may be desirable. To enable simultaneous MIC and bacterial species detection in a simple and rapid miniaturized format, a 3D-printed frame was designed for a multi-sample millifluidic dip-slide device that combines panels of identification culture media with a range of antibiotics (Ampicillin, Amoxicillin, Amikacin, Ceftazidime, Cefotaxime, Ofloxacin, Oxytetracycline, Streptomycin, Gentamycin and Imipenem) diluted in Muëller–Hinton Agar. Our proof-of-concept evaluation confirmed that the direct detection of more than one bacterium parallel to measuring MIC in samples is possible, which is validated using reference strains *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 10145, and *Staphylococcus aureus* ATCC 12600 and with mastitis milk samples collected from Reading University Farm. When mixtures were tested, a MIC value was obtained that reflected the most resistant organism present (i.e., highest MIC), suggesting it may be possible to estimate a minimum effective antibiotic concentration for mixtures directly from samples containing multiple pathogens. We conclude that this simple miniaturized approach to the rapid simultaneous identification and antibiotic susceptibility testing may be suitable for directly testing agricultural samples, which is achieved through shrinking conventional tests into a simple "dip-and-incubate" device that can be 3D printed anywhere.

Keywords: microfluidic microbiology; antimicrobial resistance; dip slide; minimum inhibitory concentration; 3D printing; miniaturized microbiology

Citation: Diep, T.T.; Bizley, S.; Edwards, A.D. 3D-Printed Dip Slides Miniaturize Bacterial Identification and Antibiotic Susceptibility Tests Allowing Direct Mastitis Sample Analysis. *Micromachines* **2022**, *13*, 941. https://doi.org/10.3390/mi13060941

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1. Introduction

Rising antimicrobial resistance remains one of the biggest global threats to public health, animal health, and the environment. For example, within an agricultural setting, bovine mastitis is a major challenge to the dairy industry both economically through reduced milk production and affecting animal well-being. Antibiotic use to treat mastitis is associated with increasing antibiotic resistance. In addition, there is a great diversity of both pathogens and commensal microbial communities present in bovine mastitis [1,2]. Recent studies have indicated that microbes interacting in species and communities have contributed to individual bacterial growth and their subsequent evolution of antibiotic resistance [3,4], although the relevance of their resistance regarding selection in the microbiome is still unclear [5]. This highlights the importance of exploring the association between

host–pathogen, pathogen–pathogen and pathogen–commensals, including the positive or negative impact of antibiotics on the natural microbiota [6,7]. One way to improve this understanding in the face of microbial complexity is to scale up our ability to measure antibiotic sensitivity.

Microbiology testing remains central, contributing to diagnosis and the effective treatment of infections, and surveillance to guide empirical antibiotic selection is important as well. Measuring minimum inhibitory concentration (MIC) using antibiotic susceptibility testing assays remains vital, and it is included in many standardized and clinical reference methods, with more accuracy compared to disk diffusion [8]. The MIC is still an important value for making clinical decisions. Combined with a pharmacokinetic-pharmacodynamic profile, MIC values guide the selection of the effective dose for an antibiotic [9,10]. Analysis of MIC remains critical for setting breakpoint threshold concentrations that define resistance vs. susceptibility, underpinning the surveillance of resistance levels among bacteria important to human and animal health [11]. Monitoring MIC shows new bacterial resistance trends as they emerge, and they can be used to modify clinical doses administered to achieve a required therapeutic concentration in patients or animals [12]. MIC measurements form a vital quality control and standardization tool between different laboratories across the world. Every year, MIC values are reported to reference agencies including CLSI, EUCAST, and BSAC and are used to define standards for clinical and veterinary laboratories and to assess global microbiological susceptibility trends [5,13].

MIC is measured by a range of antibiotic susceptibility assays in vitro, including broth microdilution, agar dilution, and antimicrobial gradient methods such as E-test (bioMerieux, Marcy–L'Etoile), MIC Test Strip (Lio-filchem Inc., Waltham, MA, USA), M.I.C. Evaluator (Oxoid, Basingstoke, UK) and Ezy MIC Strip (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Many automated systems adapt these methods for higher throughput. These are classically conducted as routine methods within a microbiology laboratory, but they are associated with high costs, a need for skilled workforce with careful quality control systems and substantial labor time [14]. The interpretation of MIC results can be influenced by several factors including the concentration of bacterial inoculum, the way the concentration of antibiotics is prepared, and the quality of media. The current approach detects MIC for a single bacterial isolate and as a result typically takes 2–3 days to complete and analyze as the pure strains need to be isolated after overnight culture before performing identification and then finally antibiotic susceptibility to determine MIC. In well-resourced situations, rapid identification (e.g., MALDI-TOF) combined with rapid susceptibility testing systems can reduce this to 1–2 days. The impact of this for people without rapid identification or susceptibility testers is that it delays the physician or veterinary surgeon's decision making, resulting in a possible delay in patient treatment. Alternatively, antibiotics are used empirically without testing. In addition, with current methods, there are a number of other factors that reduce/restrict the efficiency of the testing process, including the following: antibiotic panels of testing from commercial suppliers are not customizable, significant time is required for agar media preparation, and supply chain challenges remain regarding the import of specific antibiotics in some countries. A separate weakness of current testing protocols is a focus on single isolates that may not reflect the progress of any infection caused by a combination of multiple bacterial strains, either multiple pathogens or a single pathogen growing amongst commensal organisms that may influence antibiotic responsiveness [15].

To address the high level of antibiotic resistance, a variety of methods have been developed and commercialized such as clinically oriented automated systems including VITEK 2® [16] and MALDI-TOF VITEK MS® systems [17]. Several factors must be considered when developing suitable methods including time saving, cost saving, reliability and robustness of the method, alongside convenience for the end-user. The majority of molecular biology-based methods offer the rapid detection of specific targets including identification and a panel of common resistance genes, alongside the expansion of next-generation sequencing that has improved our understanding of microbiome and the resistome. We still

rely heavily on phenotypic assays, and these continue to focus on single strains, yet the importance of understanding the response of mixed microbial populations to antibiotics is driving the development of direct sample testing methods.

Recently, the use of digital cameras for timelapse imaging has contributed to improved analytical microbiology methods such as the early detection of antimicrobial resistance (AMR). Based on the falling cost and rapidly improving performance of digital cameras driven by consumer products (smartphones), the process of bacterial growth and response to antibiotics can be recorded without requiring large colonies clearly visible to the eye after overnight growth. Many miniaturization efforts focus on the microscopic analysis of growth exploiting digital cameras for time-resolved microscopy [18,19]. Others have directly imaged bacterial particles in liquid using digital cameras imaging fluorescence [20] or scatter [21]. Conventional culture methods have been improved through digital imaging, giving us a far more detailed understanding of colony growth on solid media [22]. These innovations have reached clinical microbiology guidelines, with EUCAST protocols allowing disc-diffusion antibiotic susceptibility to be recorded in as little as 4 h of culture [23].

At the same time that high-performance cheap digital cameras permit smaller colonies to be detected earlier, additive manufacturing methods such as 3D printing have become inexpensive and accessible, with fused-filament deposition methods cost-effective for producing routine microbiology labware [24]. Applying 3D printing to rapid custom microbiology labware production, we designed a new frame to create dip slides that combine two main functional goals: (1) directly identify and (2) determine the antibiotic MIC of microbes from samples. By shrinking solid culture format by >100-fold, a set of 6 mm well diameters in each dip slide frame uses far less media but still allows single colonies of bacterial species to grow and be identified. The detection of smaller colonies on these miniaturized versions of Petri dishes is aided by digital imaging using inexpensive cameras (e.g., smartphone, compact consumer camera), thereby avoiding expensive laboratory scanning instruments. The 3D-printed frame holding multiple antibiotic concentrations in agar is easy to prepare and has a fully customizable panel, making it easier to use compared to the current agar dilution methods. The aims of this study are to firstly optimize the 3D-printed frame for making dip slides for the detection of reference bacterial strains and to analyze the antibiotic susceptibility profile, to secondly observe the kinetics of bacterial growth on the different agar surfaces, and finally to evaluate the analysis of bacteria in bovine mastitis samples as a proof-of-concept application.

2. Materials and Methods

2.1. Experimental Approach

The design of the 3D printed frame used in this study includes two parts to identify bacterial species with two 35 mm × 7 mm rectangles; and to determine MIC, we used two rows of 6 mm diameter round wells. All designed files were published as open source models for customization or 3D printing by anyone [24]. The two rows of 10× round wells include up to 10 antibiotic concentrations, which are typically prepared as doubling dilutions. The size of the round-shaped well was >150 times smaller than current conventional Petri dishes (Figure 1), allowing each sample to be tested on a large panel of conditions.

2.2. Bacterial Strains, Mastitis Samples, Media, and Measurement of MIC

Bovine mastitis samples were collected from the Centre for Dairy Research (CEDAR) at the University of Reading. All samples were transported to the laboratory and processed/analyzed on the collection day. Milk samples were diluted at 3 serial dilutions until $1{:}10^{-3}$, and 100 µL of these dilutions was applied to streak out onto the CHROMagarTM Mastitis agar plates prepared following manufacturers guidance (CHROMagar™, Paris, France) to identify the bacteria. Antibiotic susceptibility tests were performed by agar dilution on Muëller–Hinton Agar (MHA) (Sigma). All reference strains and isolates, including *K. pneumoniae* ATCC 13883, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 10145, *S. aureus* ATCC 12600, and isolates from mastitis samples such as *Staphylococcus aureus*,

E. coli, *Pseudomonas* spp., *Klebsiella* spp., *Streptococcus uberis*, and *Streptococcus agalactiae* were inoculated overnight to reach at 10^8 CFU/mL in Muëller–Hinton Broth (MHB) (Sigma for microbiology, Dorset, UK). Conventional MIC measurement was performed with CLSI guideline (CLSI M100-ED31:2021) and also by the E-test methods. Examples of different specific culture media including MacConkey agar (MC), Baird Parker Agar (BP), Muëller Hinton Agar (MHA) (Sigma Aldrich, Dorset, UK), and CHROMagar^{TM} Mastitis (CHROMagar™, Paris, France) were used to identify bacteria.

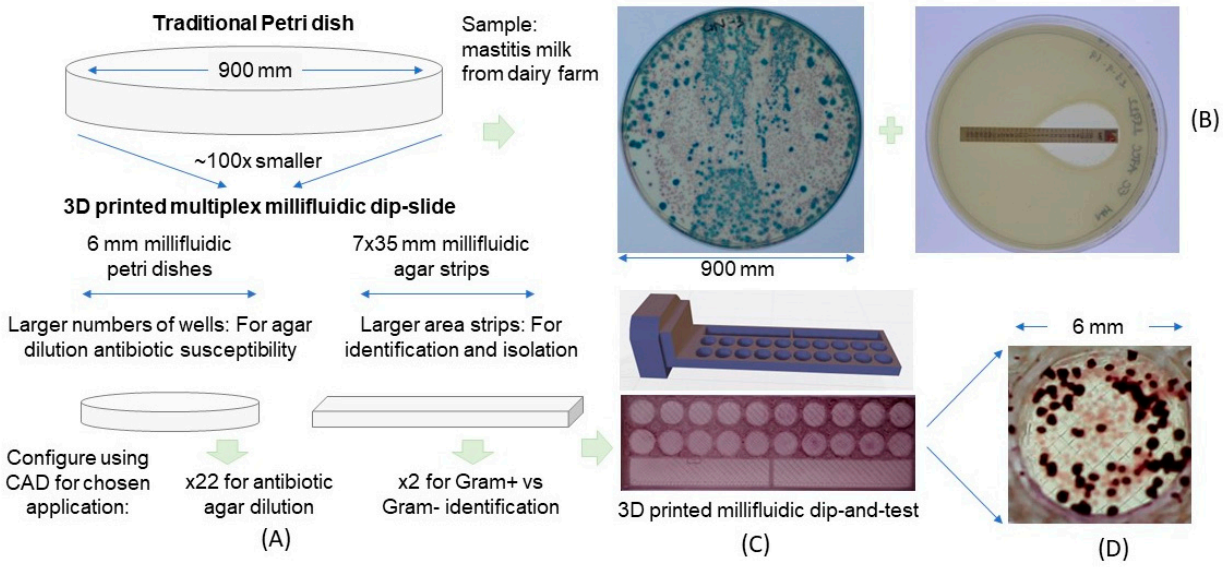

Figure 1. Shrinking Petri dishes. Miniaturized 3D-printed arrays for solid microbiological media. (**A**) Illustration of how a conventional Petri dish can be shrunk into a frame designed with different areas depending on required function. Smaller circular wells are used for antibiotic agar dilution, whereas longer strips are better suited to identification and colony isolation. (**B**) Examples of identification of bacteria and antibiotic susceptibility testing using a conventional Petri dish. (**C**) CAD illustration and photograph of 3D-printed frame for customized multiplex dip slide. (**D**) Zoomed-in image showing small bacterial colonies grown on individual wells of the 3D printed dip-slide frame.

2.3. Bacterial Identification and MIC Measurement Using 3D-Printed Dip Slides

2.3.1. Preparation of 3D Printed Dip-Slide Frame

The Standard Tessellation Language (STL) design of the frame dip slide is available online [24], the frame dips slide were 3D printed, sterilized with 70% alcohol and dried before use. CHROMagar™ Mastitis (CHROMagar™, Paris, France) was prepared according to the manufacturer's instructions and added to long rectangles to identify bacterial species. Muëller–Hinton (MH) Agar with triphenyl tetrazolium chloride (TTC) dye added to make colonies appear more strongly in digital photographs was added with serial dilutions of antibiotics to determine MIC. The antibiotic powders of Ampicillin, Amoxicillin, Amikacin, Ceftazidime, Cefotaxime, Ofloxacin, Oxytetracycline, Streptomycin, Gentamycin and Imipenem were purchased from Sigma Aldrich (Dorset, UK). The range of 9 antibiotic concentrations prepared in MH agar for testing was from 0.125 to 32 µg/mL for each antibiotic in a doubling dilution series.

2.3.2. Detection of Bacterial Species and Determining MIC Using 3D-Printed Dip Slides

After collecting, milk samples were diluted into two serial 10-fold dilutions and considered as testing samples. The dip slides were directly dipped into this diluted milk, kept submerged in the samples for 1 min, and removed, allowing excess sample to drain off the dip slide. Finally, the dip slide was placed in a sealed box to prevent drying out and incubated at 37 °C for 12 h (Figure 2). The interpreted results were based on

the bacterial growth on two types of agar media. Firstly, the colony appearance on two types of commercial chromogenic and selective agars was noted for species identification—Gram-negative and Gram-positive, with different colored colonies identified following the manufacturer's guidance. Alongside identification, a panel of antibiotic concentrations in agar was used for agar dilution determination of MIC (Figure 2). MIC results were recorded as the lowest concentration of antibiotic that inhibits the growth of bacteria. Note that the exact volume of sample deposited following dipping was not known; therefore, colonies were not counted to determine bacterial cell density. Thus, although the number of colonies could be counted visually by eye or through image analysis of digital photographs, the smaller area of solid media means that the range of concentrations that can be easily counted is smaller than for a conventional Petri dish.

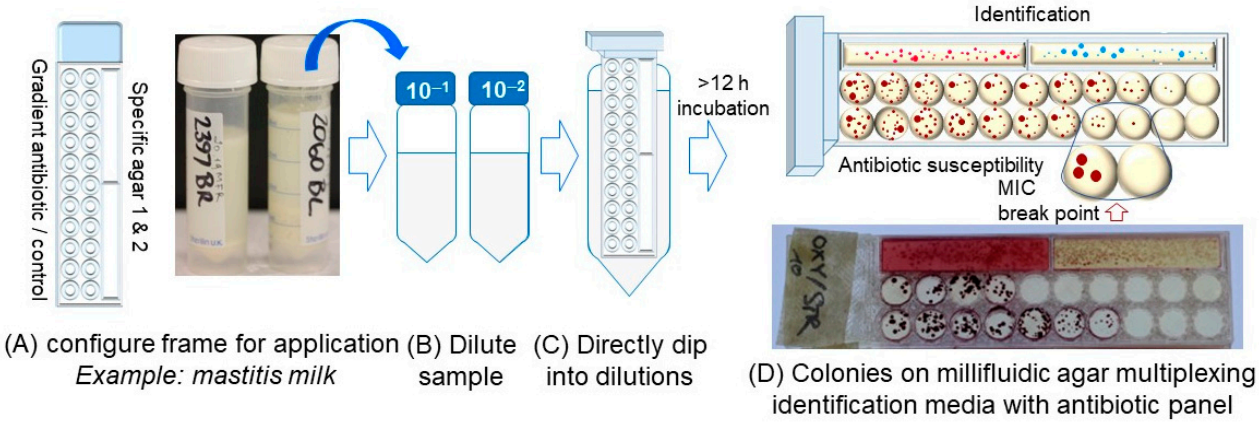

Figure 2. Dip-slide testing method. (**A**) Configure frame for application and mastitis milk samples. (**B**) Dilute sample into 2 fold, (**C**) Directly dip frame into dilutions, (**D**) Observe the results on frame.

Muëller–Hinton Agar was used for the determination of MIC according to standard agar dilution methodology, and CHROMagarTM Mastitis (CHROMagar™, Paris, France) was used for identifying species. The colonies with specific colors helped to identify specific bacterial species, and at the point with and without bacterial colonies, growth was used to determine MIC breakpoint (Figure 2).

2.4. Time-Lapse Imaging to Check Kinetics of Bacterial Growth on Millifluidic Solid Microbiological Media

With the same preparation as that of the dip slide, MH, MacConkey agar, and CHROMagarTM Mastitis (CHROMagar™, Paris, France) were applied to observe the kinetics of bacterial growth for *E. coli* ATCC 25922. The Raspberry camera 2.0 and Python code was used to take the photo, and ImageJ was used to build the time-lapse of bacterial growth. The distance from camera to 3D frame was 10.5 cm, and one cover with 3.5 cm height was used to prevent the agar from drying during time-lapse imaging. In other experiments, a digital Canon EOS 1300D with a Canon EF-S 60 mm f/2.8 Macro USM Lens was used to capture images representing the bacterial morphology on agar on the dip slides.

2.5. Comparison of MIC Breakpoints with E-Test Trip and Broth Microdilution in a Single Strain and a Mixture of Bacterial Strains

We compared our frame dip slide to broth microdilution methods. To do so, the density of inoculum was standardized at 10^8 CFU/mL equivalence; i.e., McFarland 0.5 was used for testing with the frame dip slide and broth microdilution method. The panel of antibiotic and dye was used the same for frame and broth microdilution methods; the final volume for each test was 200 µL in 96-well plates. Both the frame dip slide and microwell were kept at 37 °C overnight for endpoint analysis (12–16 h). The MIC was read at the points where antibiotics stopped the growth of bacteria. The whole protocol to evaluate the novel dip-slide test was mapped in Figure 3.

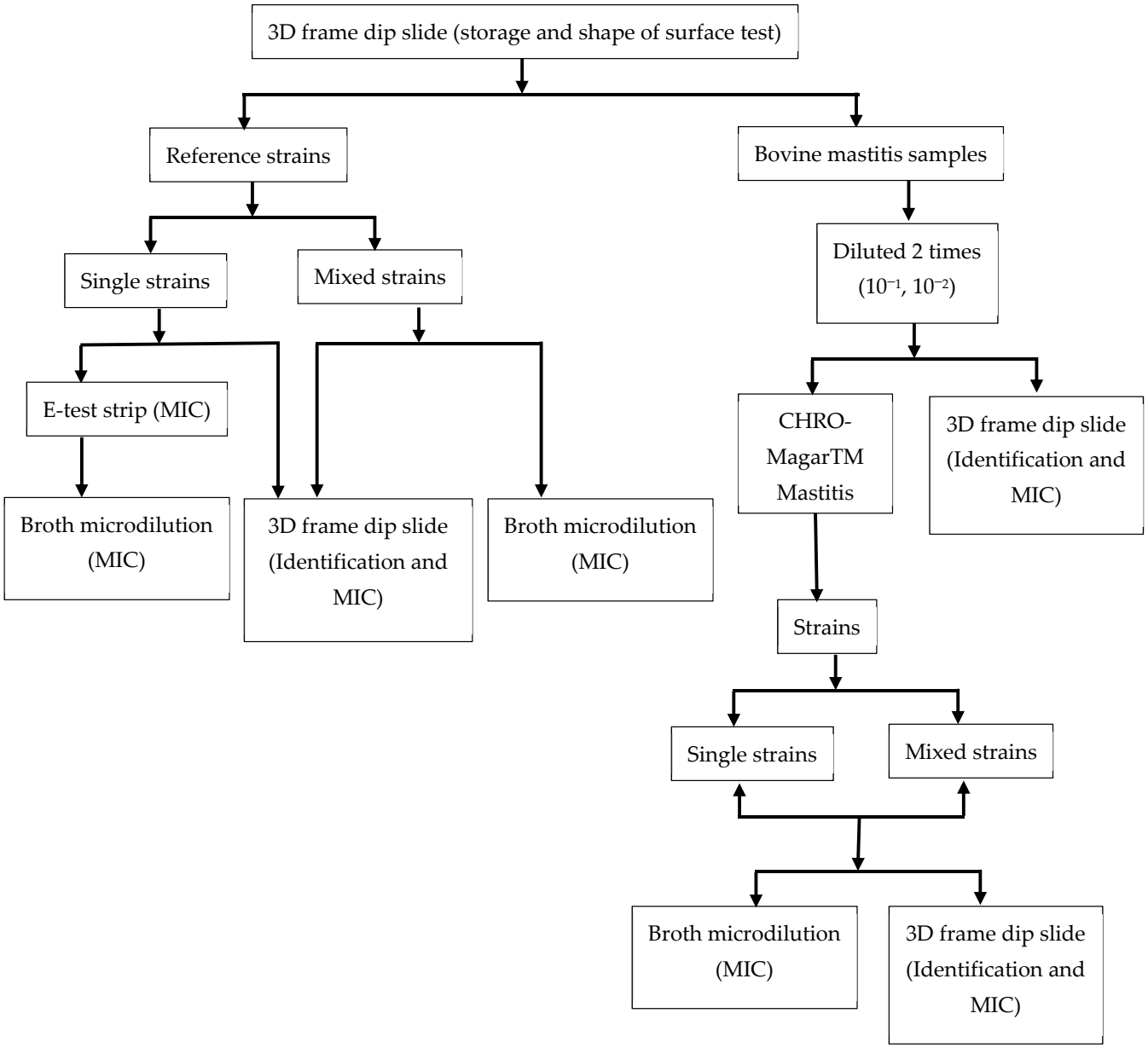

Figure 3. Flowchart showing isolation, identification and sensitivity testing for reference and mastitis strains.

3. Results

3.1. Use of 3D-Printed Multiplex Dip Slide for Bacterial Identification and Antibiotic Susceptibility Testing

The 3D-printed frame allowed us to combine two types of solid media on a single dip slide. Longer wells provided enough space to identify colonies of bacterial species on specific chromogenic agar for Gram negatives or Gram positives simultaneously. Alongside this on the same device, agar dilution antibiotic susceptibility testing was possible. Even with only 6 mm diameter wells, single separate colonies were visible on these tiny solid media wells, with a similar appearance to a conventional Petri dish culture (Figure 1). Customization of the CAD design allows the user to decide the type of agar media required for testing to match the test purpose in the laboratory. Colonies visible on the miniaturized 6 mm wells could be counted by eye or from digital images, but this device was not designed for colony counting. Whilst we found testing very repeatable, the dip-slide format does not deposit a precise known volume onto the agar. Here, we focused on

mastitis milk sample testing and adapted frames for two different identification agars provided in the CHROMagar™ Mastitis product (CHROMagar™, Paris, France). Using this frame, <1 mL of each type of agar is needed instead of 20 mL of agar for a standard 100 mm Petri dish. The MIC agar dilution wells require just 140 µL of media per slide, which is >70 times less than standard test tube agar dilution protocols and >150 times less than Petri dish, alongside a major reduction in incubator space.

3.2. Characteristics of Multiplex Multifluidic Dip Slides

By dipping directly frame into the sample (e.g., diluted milk) and incubation at 37 °C (Figure 2), the results were able to be reported in just 8 h for *E. coli* and around 10–12 h for other bacteria such as *Staphylococcus* spp. and *Klebsiella* spp. (Figure 4). Bacterial colonies grown on the dip slide appeared comparable to those grown on a Petri dish for a wide range of agar types. However, it was significantly easier to record smaller bacterial colonies with a digital camera when using media that contained a supplement of TTC dye rather than Muëller–Hinton agar without dye, where the yellow-gray colony color was only weakly visible on the background of the agar color itself (Figure 4A).

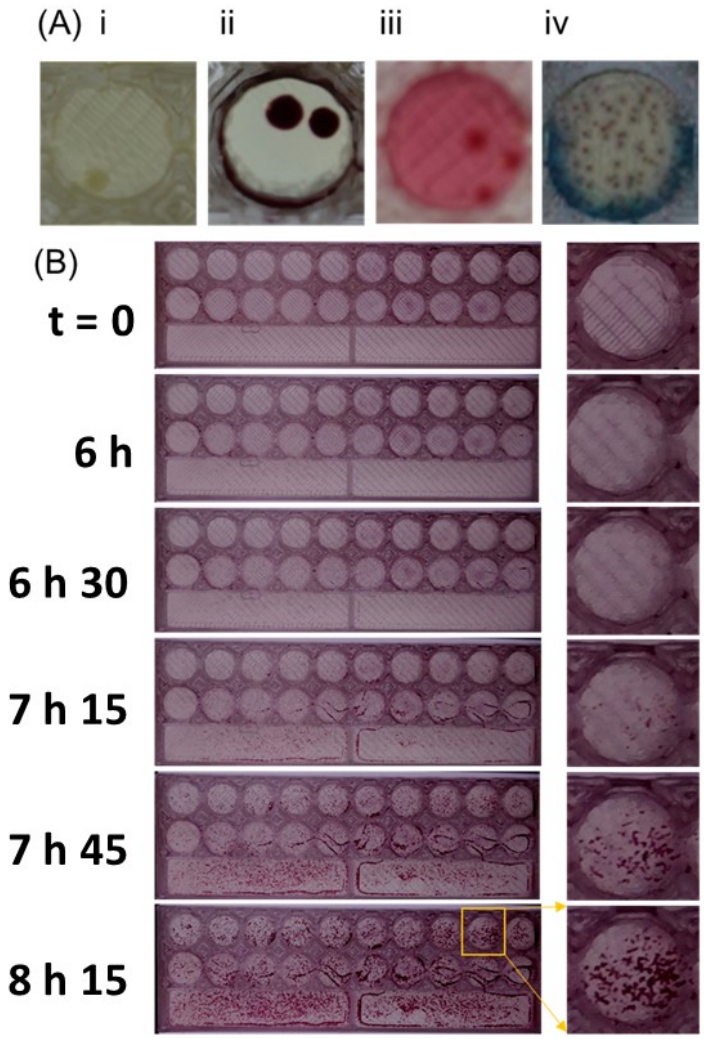

Figure 4. Different media appearance and kinetics of growth on millifluidic solid microbiological media. (**A**) The appearance of colonies on 6 mm 3D-printed discs containing the following agar types: Muëller–Hinton Agar (MHA) (i), MHA with triphenyl tetrazolium chloride (TTC) dye added (ii), MacConkey (iii), and CHROMagar™ Mastitis Gram⁻ (iv). (**B**) Selected timepoints from time-lapse analysis of colony growth, showing the emergence of colonies around 7–8 h on MHA with TTC.

To monitor speed of colony appearance, a Raspberry Pi equipped with the v2 camera module was used to capture images every 10 min. This time-lapse analysis showed that the results of MIC can be interpreted around 7–8 h for *E. coli* (Figure 4B) and more than 10 h for other bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*. This means that the results of MIC can be interpreted during 7–8 h compared to routine methods needed overnight (Figure 4B).

3.3. Detection of Individual Bacteria Isolates and Their Antibiotic Resistance Profile

Three-dimensional (3D)-printed dip slides with sets of miniaturized 6 mm diameter dip-slide wells plus rectangular identification strips were compared to conventional broth microdilution for the capacity to detect individual strains and measure their antibiotic susceptibility. Antibiotics such as Ampicillin, Amoxicillin, Amikacin, Ceftazidime, Cefotaxime, Ofloxacin, Oxytetracycline, Streptomycin, Gentamycin and Imipenem were used for this purpose, with Figure 5 showing images with four representative antibiotics. There was an agreement of MIC results between our dip slide to broth dilution methods.

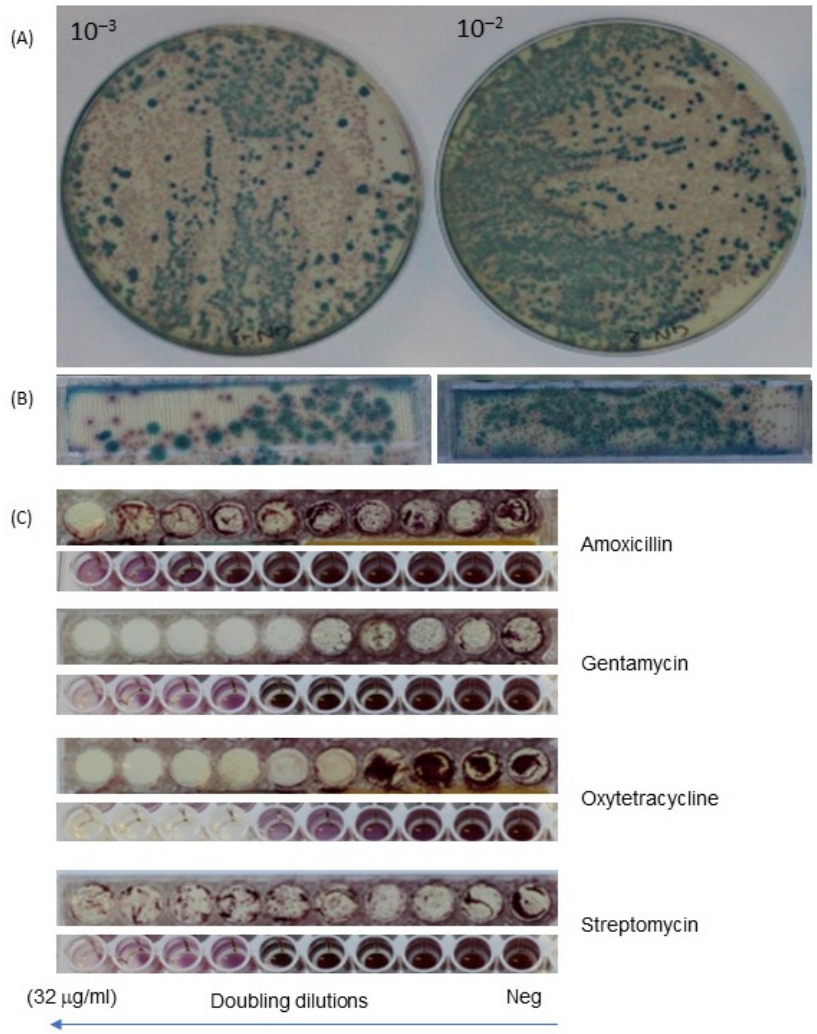

Figure 5. Comparison of identification and determination of MIC through frame dip slide vs. Petri dish combined with broth microdilution on mastitis milk sample. (**A**). Bacteria (*E. coli*—pink colonies, *Klebsiella*—blue colonies) grown on CHROMagar™ Mastitis (CHROMagar™, Paris, France) at two dilutions on Petri dish, (**B**) Bacteria grown at two dilutions on frame, (**C**) Comparison of the results of MIC between frame (upper image for each antibiotic) and broth microdilution (lower image).

Next, bacterial isolation was compared between the agar dish and miniaturized dip slides with specific agar media—Mackonkey agar (MC) and CHROMagarTM Mastitis (CHROMagar™, Paris, France). Notably, the data indicated 100% accordance of individual strain using different agar media for testing the bacterial growth. The results showed *E. coli*—pink colonies and *Klebsiella* spp.—blue colonies grown on CHROMagarTM Mastitis (CHROMagar™, Paris, France) were the same results on the frame and agar dish (Figure 5).

As expected, although the overall pattern of antibiotic sensitivity was the same, there were some minor disagreements in MIC results between the dip slide and conventional broth dilution for some strain/antibiotic combinations. For example, Gentamycin differed by one doubling dilution of MIC (Figure 5). However, this is common for MIC determination and can result from different interpretations of intermediate growth, where it can be hard to decide which dilution constitutes inhibition vs. growth. For example, when capturing the photo, some small colonies can be seen on agar by eye, but these are not obvious on the digital photo, whereas the microplate shows intense coloration from microbial growth at this concentration, possibly resulting in the difference seen in the MIC of one doubling dilution. Despite the small surface agar (6 mm), bacterial colonies have still been separately seen, allowing the inhibition by antibiotics to be seen. This is important as after performing MIC, many resistant strains will be kept for further testing (e.g., for surveillance or reporting) research in the future such as genotype and prediction of resistant trends of bacterial collection. With such purpose, an individual colony was collected and kept in the biobank. There was no difference in picking individual colonies between tube test, Petri dish or dip-slide frame (Figures 5 and 6). For example, when comparing the MIC breakpoint and the *E. coli* growth on specific agar—MC and CHROMagarTM Mastitis (CHROMagar™, Paris, France), the data showed that the *E. coli* grew on MC and CHROMagarTM Mastitis (CHROMagar™, Paris, France) with typical pink colony, and its MIC to Oxytetracycline was 1 μg/mL. At this concentration, there was no bacterial growth on this concentration. However, for Streptomycin and Amoxicillin, the MIC breakpoint was over 32 μg/mL because *E. coli* kept growing at this concentration (Figure 6). The same results have been found on the broth dilution methods with the same strains and antibiotics. With the current MIC methods, strains needed to be isolated and identified, and the purity needs to be checked before performing MIC. Alongside that, an extra test tube—just bacteria without antibiotic—was needed to conduct parallel with MIC. The main purpose for doing this is to make sure the right clone of bacteria was relevant to its MIC. Our frame, however, does not need to perform this step because resistant strains still grew on the agar media, and it was easy to collect the right clone for storage in terms of further research.

3.4. Direct Testing of Mixtures of Bacteria and Their Antibiotic Resistance Profile

In reality, bacteria live in their niches or community, not existing as pure individual strains, and this can affect both response to antibiotics and the measurement of antibiotic MIC. Many samples contain mixtures of microbes; it is rare to find a sample with only one single species or strain, although clinical samples often contain one dominant pathogen that can be readily identified, which forms the basis for established conventional microbiology testing. A second reason for testing mixtures is to reduce the time-to-result by directly testing samples without initial overnight culture and colony isolation. Therefore, it remains important to explore if antibiotic susceptibility of mixtures of pathogens could be measured. To do this, we tested mixtures of pathogens isolated from mastitis milk as well as reference strains, using a multiplex dip slide compared with broth microdilution as well as testing mixtures of reference strains as control groups. With our dip-slide frame, there were always two parts: identification and MIC breakpoints. On the identification panel, the aim was to identify different bacteria present as single colonies present on specific types of identification agar. However, the MIC was not identical if a single strain was compared to two mixed strains. To prove this concept, we performed MIC for each single strain (Table 1) and then mixed them at equal cell concentrations and performed MIC again for the mixture. In the experiment of mixture strains, the MIC results always showed

the highest concentration of antibiotic resistance. For example, the MIC of amoxicillin of *S. aureus* isolated from milk samples was 16 μg/mL and was 32 μg/mL for *Pseudomonas aeruginosa* isolated from milk as well (Figure 7). When combing two strains into a mixture, the results showed an MIC of 32 μg/mL for both strains together (Table 2). These findings implicated that the final MIC value was the highest concentration of pathogens in the mixture. However, to conclude resistance or susceptibility for individual species or isolates, we need to follow guidelines such as CLSI or EUCAST that specify single colonies must be picked prior to testing.

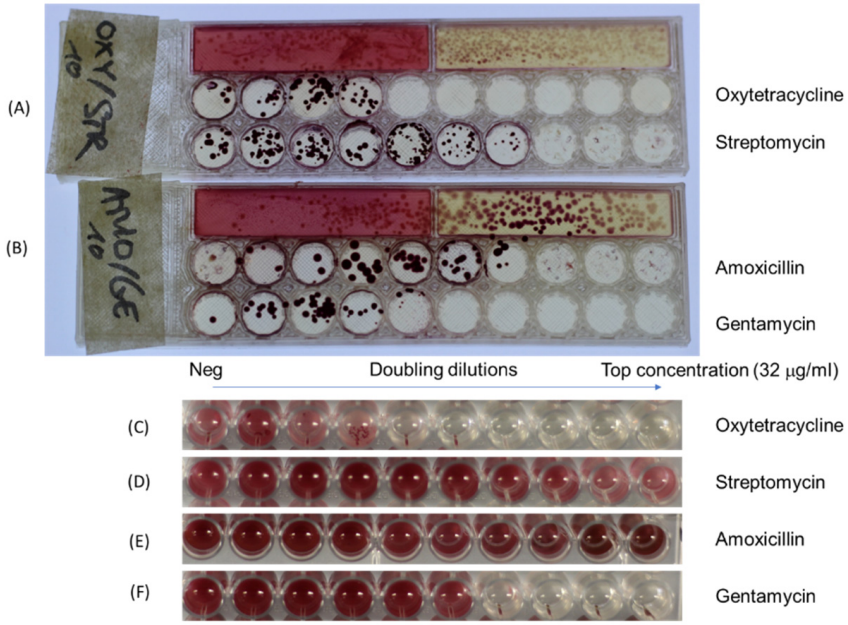

Figure 6. Comparison of frame dip slide and broth dilution methods to determine *E. coli* isolated from the bovine sample and its MIC. (**A**) The results of dip-slide tests of *E. coli* on MC/CHROMagar™ Mastitis Gram negative and its MIC for Oxytetracycline and Streptomycin, (**B**) The results of *E. coli* on MC/CHROMagar™ Mastitis Gram negative and its MIC for Amoxicillin and Gentamycin, (**C**) The results of *E. coli* on broth microdilution of Oxytetracycline, (**D**) The results of *E. coli* on broth microdilution of Streptomycin, (**E**) The results of *E. coli* on broth microdilution of Amoxicillin, (**F**) The results of *E. coli* on broth microdilution of Gentamycin.

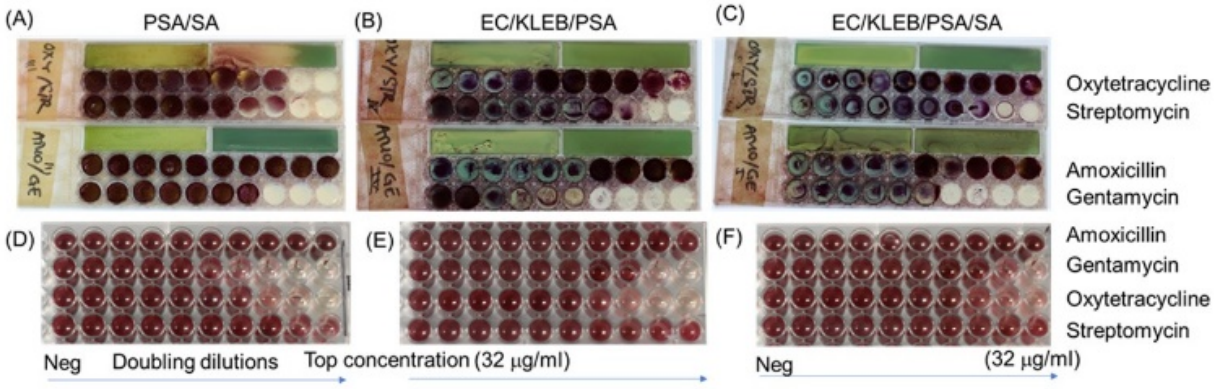

Figure 7. Antibiotic susceptibility by agar dilution for mixtures of microbes, simulating multiple organisms present in some samples. Mixtures of the following strains were prepared and split for testing in parallel using 3D-printed multiplex millifluidic dip slides (**A**–**C**) vs. conventional microplate broth microdilution (**D**–**F**). Naming of microbial species: *Pseudomonas aeruginosa* (PSA), *Staphylococcus aureus* (SA), *E. coli* (EC), *Klebsiella* spp. (KLEB).

Table 1. Comparison frame dip slide vs. broth microdilution to individual strains.

Testing Samples	Methods	Antibiotic MIC (mg/mL)								
Reference Strains		**AMP**	**AMO**	**AMI**	**CEF**	**CEFO**	**OFL**	**OXT**	**STR**	**GEN**	**IMI**
Escherichia coli ATCC 25922 (EC)	Frame	4	>32	0.5	16	>32	2			1	0.125
	MIC broth	4	>32	0.5	16	>32	2			1	0.125
	E—strip test	4								1	0.125
Klebsiella pneuminae ATCC 13883 (KB)	Frame	>32	>32	1	16	>32	2			1	0.5
	MIC broth	>32	>32	1	16	>32	2			1	0.5
	E—strip test	>32								1	0.5
Pseudomonas aeruginosa ATCC 10145 (PSA)	Frame	>32	>32	2	16	>32	2			0.5	1
	MIC broth	>32	>32	2	16	>32	2			0.5	1
	E—strip test									0.5	1
Staphylococcus aureus ATCC 12600 (SA)	Frame	<0.125	>32	1	16	>32	2				
	MIC broth	<0.125	>32	1	16	>32	2				
	E—strip test	0.032									
Direct from milk mastitis samples											
19.02 MRF (EC)	Frame		>32					1	>32	4	
	MIC broth		>32					1	>32	4	
19.03 MRF (*Klebsiella* sp.—K)	Frame		>32					2	16	4	
	MIC broth		>32					2	16	4	
19.07 MRF (SA)	Frame		>32					>32	>32	4	
	MIC broth		>32					>32	>32	4	
19.08 MRF (SA)	Frame					16		8			
	MIC broth					16		8			
19.09 MRF (SA)	Frame		>32					16	>32	8	
	MIC broth		>32					16	>32	8	
19.09 MRF (*Klebsiella* sp.)	Frame	>32	>32	>32	16	4	2				
	MIC broth	>32	>32	>32	16	4	2				
19.10 MRF (EC)	Frame		>32					1	>32	2	
	MIC broth		>32					1	>32	2	
19.10 MRF (*Streptococcus uberis*)	Frame		>32			16					
	MIC broth		>32			16					
19.11 MRF (*Streptococcus agalactiae*)	Frame		8			16					
	MIC broth		8			16					
19.14 MRF (PSA)	Frame		>32			>32		1			
	MIC broth		>32			>32		1			
19.15 MRF (*S. aureus*—SA)	Frame		16			>32		2			
	MIC broth		16			>32		2			
19.16 MRF (EC)	Frame		16			8		2	>32	>32	
	MIC broth		16			8		2	>32	>32	
19.16 MRF (SA)	Frame		>32					1	8	16	
	MIC broth		>32					1	8	16	
19.17 MRF (*Streptococcus agalactiae*)	Frame		8			16					
	MIC broth		8			16					
19.17 MRF (SA)	Frame		>32					8	16	4	
	MIC broth		>32					8	16	4	
20. 20 MRF (*S. aureus*—SA)	Frame		8			32					
	MIC broth		8			32					
20. 20 MRF (PSA)	Frame		>32					>32	>32	8	
	MIC broth		>32					>32	>32	8	

Interestingly, when observing the interaction of bacterial mixtures to antibiotic resistance, the results showed that there was no significant difference between using the frame dip slide and broth dilution, even performing on reference strains or isolates from bovine mastitis samples. We also tested the frame with *Streptoccocus* species and found an agreement of bacterial growth on two methods. The small compact design makes this ideal for use for obligate anaerobes within an anaerobic cabinet and incubator. Furthermore, the dip slide can be applied for both single strain and mixture strains (Tables 1 and 2). Table 1 summarizes the MIC results for single reference and pure isolated strains; the abbreviation of "MRF" for Mastitis Reading Farm was used to name individual isolates from milk samples from cows suffering from mastitis. In Table 2, the MIC results observed for dip slides vs. conventional methods are presented for various mixtures of different microbial strains and for directly tested mastitis milk samples.

Table 2. Measure the impact of mixtures pathogens to antibiotic resistance on reference strains and mastitis strains.

Testing Samples	Methods	Antibiotic MIC (mg/mL)									
		AMP	AMO	AMI	CEF	CEFO	OFL	OXT	STR	GEN	IMI
Reference Strains											
EC/SA/KB (Reference strains)	Frame	>32	>32	2	16	>32	2				
	MIC broth	>32	>32	2	16	>32	2				
EC/PSA/KB (Reference strains)	Frame	>32	>32	2	16	>32	2	>32	>32	>32	
	MIC broth	>32	>32	2	16	>32	2	>32	>32	>32	
EC/SA/PSA/KB (Reference strains)	Frame	>32	>32	2	16	>32	2	>32	>32	>32	
	MIC broth	>32	>32	2	16	>32	2	>32	>32	>32	
Strains Isolated from mastitis samples											
19.15 MRF (SA)/20.20 MRF (PSA)	Frame		>32					>32	>32	8	
	MIC broth		>32					>32	>32	8	
19.17 MRF (EC)/19.09 MRF (KB)/20.20 MRF (PSA)	Frame		>32					>32	>32	>32	
	MIC broth		>32					>32	>32	>32	
19.17 MRF (EC)/19.15 MRF (SA)/20.20 MRF (PSA)/19.09 MRF (K)	Frame		>32					>32	>32	>32	
	MIC broth		>32					>32	>32	>32	
19.17 MRF (EC)/19.09 MRF (K)	Frame		>32					16	>32	>32	
	MIC broth		>32					16	>32	>32	
19.17 MRF (*Streptococcus agalactiae*) + 20.20 MRF (*S. aureus*—SA)	Frame		8			>32					
	MIC broth		8			>32					
19.10 MRF (*Streptococcus uberis*) + 19.15 MRF (*S. aureus*—SA)	Frame		>32			>32					
	MIC broth		>32			>32					
Direct from milk mastitis samples											
19.07 MRF	Frame		>32					>32	>32	4	
	MIC broth		>32					>32	>32	4	
19.09 MRF	Frame	>32	>32	>32	16	4	2	16	>32	8	
	MIC broth	>32	>32	>32	16	4	2	16	>32	8	

4. Discussion

We characterized a simple method to make and use a 3D-printed frame design that combines multiple miniaturized agar wells into a convenient dip slide. We asked if pathogens can be identified alongside MIC determination in one direct "dip-and-test" device. The proof-of-concept results presented here show it is feasible for bacterial species to be identified on miniaturized wells of chromogenic media alongside agar dilution for antibiotic susceptibility testing, with good agreement to conventional Petri dish methods. We also show that these dip slides can simultaneously measure mixed samples containing up to four different bacteria representing more complex clinical situations where multiple organisms are present in a single sample. The overall observed MIC was similar to the inhibitory concentration for the strain within the mixture with the highest inhibitory concentration. We also demonstrate the feasibility of using these "millifluidic" multiplex dip slides for the direct testing of milk samples from cows with mastitis. From such results, we suggest that the physician or the farmer can use these rapid direct test results to select an appropriate antibiotic, reducing the risk of treatment failure if the animal is infected with

at least one resistant pathogen and avoiding the need for empirical use of broad-spectrum antibiotics without testing.

There is a trade-off in sampling simplicity achieved by miniaturization vs. absolute quantitation. A defined volume of sample can be plated on a large area with Petri dish culture, allowing precise determination of colony counts over a relatively high dynamic range (from a few colonies to several hundred on a 100 mm Petri dish). However, more space and media are required, plus skilled operation. In contrast, with the miniaturized culture area on the dip slide, it was less easy to quantify exact microbial load; however, identification and MIC determination were still possible with a much simpler procedure.

Likewise, direct testing represents a trade-off of speed to result vs. precisely measuring each organism present within the sample. Current methods to detect MIC breakpoints typically take two to three days mainly because the sample requires plating overnight, following which isolates are tested to identify organism, and finally antibiotic susceptibility tests are performed. By direct testing using a 3D-printed frame dip slide, we can reduce the turnaround time to overnight or even as little as 10 h culture. A major time-saving measure was avoiding isolating individual bacterial strains before performing the antibiotic susceptibility test; instead, the 'bulk' resistance of directly diluted samples is estimated. Whilst for some samples containing mixtures of organisms and/or pathogens, this could lead to false resistance, it is unlikely that false susceptibility will be observed, as when mixtures were tested, the MIC was always equivalent to the organism with the highest MIC, as would be expected. This reduces the risk of false susceptibility results that could lead to more significant clinical error, in which an ineffective antibiotic is selected. By combining two routine assays—the identification and determination of MIC—into one test with the frame dip slide, it should often be possible to interpret MIC through identification, as breakpoint resistance values can vary between Gram-negative vs. Gram-positive species. Digital imaging allows recording smaller colonies at earlier timepoints than conventional overnight incubation, again if the risk of missing slower-growing organisms is taken into account.

Facing relentless increases in antimicrobial resistance, many studies have investigated innovative alternative methods and technologies for antibiotic susceptibility testing and/or resistance detection including both genotype and phenotype [25]. However, most genotype-based methods are expensive (especially sequencing) and not easy to deploy widely in all laboratories. Detection methods such as LAMP and PCR can be fast but rely on the detection of specific resistance targets that can vary over time and location. For these reasons, phenotypic assays remain crucial techniques and contribute an important central role in the determination of antibiotic susceptibility for surveillance and treatment, especially the quantitation of MIC that can be used to identify breakpoint values for resistance vs. susceptibility [26]. As a result, significant advances in phenotypic assays have been continually developed such as microplate-based surface methods [27], nanoliter array [28], and multiplex fluidic chip [29]. Our 3D-printed dip slides add to this evolution, with an intermediate level of miniaturization between microfluidics and conventional large culture dishes.

Recently, significant focus has turned to the resistome and the relationship between the complex microbiome and its interaction with pathogen behavior, and whether the overall resistance of mixtures of bacteria found in the site of infection might affect the effectiveness of antibiotic treatment [13,30]. In this study, we explore the possibility of an alternative approach to the phenotype-based method that determines the MIC value directly from mixed bacterial population in a sample [3,9,31]. Whilst this may not provide the same standardized measure of antibiotic susceptibility of each individual strain present in the sample, it will be important to continue researching the link between "mixed MIC" found from direct susceptibility testing

Our assay is simpler than many microfluidic devices, and the open source 3D-printed frame design is flexible and customizable. The system is compact, allowing mobile operation especially if combined with other portable microbiology tools such as mobile

incubators [32]. Three-dimensional (3D) printing has become cheaper; we used 3D printers costing £200–£750 (Creality Ender 3 and Prusa i3 MK3) with the materials cost per frame dip slide significantly under £2 to determine simultaneously Gram-negative and Gram-positive bacterial identification plus two different antibiotics. However, in spite of being simple and inexpensive, they do need to be freshly prepared due to the limited stability of antibiotic in agar medium, and challenges remain such as scaled-up mass-production or methods to make the addition of agar to the dip slides less laborious. Future innovations should focus on exploring ways to stabilize antibiotics within such devices, permitting longer-term storage, perhaps by a combination of the dip-slide format with antibiotic discs.

5. Conclusions

With this simple miniaturization of essential phenotypic microbiology testing, the initial identification and MIC profiles can be simultaneously detected in direct samples. Three-dimensional (3D)-printed open source designs make this "millifluidic microbiology" accessible across the world. Further insight into the antibiotic susceptibility of mixed organisms in some important samples may also be gained by permitting a larger-scale direct phenotypic testing of complex samples. We showed that the 3D-printed dip slide was able to be used for identification and MIC determination for a range of bacteria. The dip frame is easy to use and can be easily transport to the field, with no extensive training or introduction to the user required. Combing the dip frame with our previously developed mobile incubator [32] will increase the portability of these tests for broader application, and it may reduce the turnaround time for samples taken a long way from microbiology testing labs. Finally, direct testing with 3D-printed dip slides may contribute to an alternative and rapid way to measure mixed microbes including the microbiome alongside pathogens and measure the antibiotic resistance profiles associated with complex mixtures.

Author Contributions: Conceptualization—T.T.D. and A.D.E. Methodology and design—T.T.D. and A.D.E. Investigation—T.T.D. Writing—original draft—T.T.D. Writing—review and editing—S.B. and A.D.E. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 4 - MOBILE INCUBATOR

Mobile incubator

This study has described the design of the mobile incubator and its application.

Full paper

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The extent of Contribution to research:

In this work, I:

1. Designed the experiment
2. Performed the test
3. Collected and analysed the data
4. Drafted the manuscript

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MicroMI: A portable microbiological mobile incubator that uses inexpensive lithium power banks for field microbiology

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ABSTRACT

Incubation at controlled temperature is a key step in culture based microbiological tests. Access to culture-based microbiological testing requires access to conventional incubators in a laboratory. Portable incubators allow microbiological testing in the field and in resource-limited settings, and can eliminate the challenge of sample transportation, minimising the chance of sample degradation. Recent studies have reported low-cost portable incubator designs suitable for field or off-grid use, but these either need an external power supply (e.g. mains AC or 12 V DC), or rely on passive heating without thermostatic control. Here we report that small inexpensive uninterruptable power supply (UPS) products manufactured for consumer electronics and powered by lithium-ion battery packs allowing thermostatic temperature control in small portable incubators that can maintain precise temperatures with or without external power. We present an open-source design for a Microbiological Mobile Incubator (MicroMI) in two sizes for field use. The MicroMI is built from simple and widely available components and is easy to set up. The open source design can be customised for different numbers of samples. The smallest and most efficient design uses a vacuum insulated food flask that allows longer operation with smaller, lower capacity UPS. The larger flight case design has space for more samples, but depletes the battery faster. The UPS maintains a typical microbiology incubation temperature for up to 24 h without external power- ideal for typical incubation needed for culture methods. The battery capacity, incubator design, and external ambient temperature all affected duration of operation without requiring external power. We validated the MicroMI by conducting classical microbiological tests using agar petri dishes, slant cultures and dip slides, and biochemical tests. We conclude the MicroMI design allows inexpensive lithium battery products to be used to simplify field microbiology and increase access to vital analytical microbiology testing.

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Introduction

Specifications table

Hardware name	MicroMI (Microbiological mobile incubator)
Subject area	• Microbiology • Environmental surveillance • Training Tool
Hardware type	Microbiological testing
Cost of Hardware	£185 – 8300 mAH capacity 12v UPS large MicroMI£120 – small MicroMI
Source File Repository	Available with the article

Hardware in context

Infectious disease including bacterial infection remains a major global public health challenge. Incubation at a controlled temperature is required for culture-based analytical microbiology, and in spite of the emergence of important new molecular methods (e.g. sequencing, PCR and mass spectroscopy), culture methods are still widely used including for sterility testing, for microbial isolation and bacterial identification through growth on solid media, often combined with biochemical and phenotype measurements, and to measure pathogen growth on differing substrates. Culture-based methods alongside functional antibiotic drug susceptibility tests remain critical for public health [7]) [11]. Some methods, such as broth microdilution antibiotic susceptibility testing, still requires culture in an incubator, and biochemical phenotyping also requires controlled temperature [10]. The first temperature-controlled incubation devices for microbiological applications were described in 1958 [4]; since then, thousands of laboratory incubator designs have been introduced and sold on the market, becoming ubiquitous inside laboratories.

Currently analytical microbiology is conducted in laboratories equipped with bespoke incubators. A major limitation of laboratory services, for example in outbreak response or global health, is slow turnaround time. Sample testing turnaround time is a key performance indicator of laboratory activities and fast results are vital for example to support hospital treatment [2,9]. In remote areas or those with limited resources, laboratory access can limit the practical use of culture-based test methods. Alongside limited access to microbiological testing labs, the transportation of specimens from local sampling sites to the reference laboratory can be a major challenge, with concerns about sample degradation during transportation if not tested soon after the sample is collected, especially for culture and phenotypic methods. Transport to laboratories can also increase cost of testing. To help address those issues, several recent studies have reported low cost mobile incubators based on widely available materials and construction methods, convenience for use in low resources environments, and for integration into automated novel technology [3,5,6,8,11].

Current innovative incubator designs have made use of heated foam ice boxes [3] or food chiller boxes [1] powered by a 12 V car battery or similar flexible power supplies. Such designs have made use of widely available and inexpensive temperature control modules, coupled to resistive heater panels and fans [11]. These are compatible with a wide range of external power sources including 12v DC and 120–240v AC, and can often be operated off-grid using existing portable power supplies. Where laboratory power supplies are interrupted, i.e. during power outages, temperature may be maintained depending on the size/insulation of the incubator, but to avoid this laboratories must be supplied by expensive backup power supplies (generator, battery). To our knowledge however, all the electrically powered incubator designs require an external power supply, whether through mains infrastructure or off-grid via battery combined with field generation. The incorporation of lithium battery power supplies into these designs has not previously been reported. Alternatives to electrically heated and thermostatically controlled incubator have been proposed, including passive heating using hot water in bottles within an insulated box [3]. Whilst effective for many applications, the temperature maintained may vary and accuracy is dependent on both ambient temperature and hot water temperature, and many microbiological testing protocols specify stricter temperature control.

The continued fall in cost and increased power:weight ratio of rechargeable batteries for example using lithium-ion cells largely driven by consumer electronics (e.g. smartphones) now provides the potential for a fully portable incubator incorporating consumer rechargeable battery packs in the design. Even more convenience is possible if an external power supply can be combined with internal batteries without user input. Here, we explored whether the latest lithium battery packs in the form of inexpensive consumer Uninterruptible Power Supply (UPS) units have sufficient power and energy capacity to maintain temperature within portable microbiological incubators in various sizes and form factors, for applications where a continuous external power supply is not available or convenient. UPS products offer internal control circuits that switch seamlessly between external mains power (e.g. 120–240v AC) vs internal battery power. Recent demand in portable consumer electronics (smartphones) has led to lightweight highly portable UPS products based on lithium battery packs with 12v output and 5000–10000 mAh capacity (at 12v) becoming widely available and costing <£100. Even larger capacity

Fig. 1. The concept of MicroMI design (*) When unplugging from external electrical supply, the MicroMI with 12 V UPS maintains temperature from 9 to 12 h, depending on incubator size, external ambient temperature, and UPS battery capacity.

(>20000mAh) products are becoming more widely available. We developed MicroMI (Microbiological mobile incubator): an inexpensive mobile incubator concept, designed to solve barriers arising from transportation of samples to microbiology laboratories, to improve turnaround time for public health microbiological testing, and to help move analytical microbiology testing into the field. We combined low cost (under £100), portable consumer UPS providing dual 12v and 5v USB outputs, with other readily available components, to create an incubator design which can be unplugged from external power and transported (Fig. 1). We found these small portable incubators maintain temperature reliably for up to 24 h without external power, long enough to complete a typical microbiology incubation between charging.

We provide here designs to build two different sized inexpensive (£125 to £185) portable incubators powered by small and lightweight consumer UPS products containing modest capacity lithium-ion batteries (manufacturer specified capacity ~ 6-8Ah at 12 V corresponding to ~<100 Wh or ~<360 kJ). The large MicroMI design has space to incubate agar plates and can be constructed entirely from off-the-shelf parts. The smaller vacuum flask design was designed to be contained within a small backpack (<15L capacity) to aid transportation and field use, yet has sufficient space to incubate 4 dip-slides or slant agar cultures; this design requires two 3D printed components in addition to off-the-shelf parts. We provide validation data indicating the power required for these two different configurations with the smaller vacuum flask requiring significantly lower power allowing it to maintain temperature for longer using the same UPS capacity.

The key design features of these mobile incubators include:

Small size (W380 × H145 × D400 mm at 2.64 kg for suitcase, and 180 × 122 × 118 mm for vacuum flask at 650 g)

3. Makes use of small portable UPS to maintain suitable microbiological culture temperatures (typically 37 °C) without external power, for example outside the laboratory or during power outages.

Simple customisable designs to make use of locally available and inexpensive (<£100) components.

This paper provides design details for both size variants and outlines the critical design parameters to facilitate customisation and use of locally-available components. We include validation data showing temperature profiles, estimated power consumption, and successful analytical microbiology testing of reference stains, real microbiological samples and different test formats including petri dish, slant tube, and dip slides. These microbiological assays are required in a range of situations where laboratory access is limited – environmental testing, point of care testing, food safety monitoring and outbreak management especially in low resources settings.

Hardware description

Overall system design

Both configurations of the MicroMI were built up from widely available off-the-shelf components, with availability from both online and local hardware suppliers

The main parameters to consider before selecting the components are: 1) Size, type and number of samples or items to incubate; and 2) Length of time required between plugging into external power supply (e.g. mains). When required, external power (12 V or AC mains) can both re-charge the internal UPS, and power the incubator to extend incubation. The ambient operating temperature will also significantly affect the battery capacity required; higher ambient temperatures will require significantly less power (and thus deplete the battery slower) to maintain 37 °C. These parameters are highlighted alongside consideration of the location and distance from field site to the local or reference laboratory, that combines with the type of

testing and location. Additionally, the laboratory space is another aspect to consider; the small scale of these incubators permits them to be set up and used in temporary testing sites or alongside other facilities (e.g., farm, health clinic). This small capacity of incubator is a good choice for laboratories with small sample numbers each day, with no need to invest in a large volume incubator for large scale testing.

The most important components are:

Uninterruptible Power Supply:

- Portable size, 12 V output, with sufficient capacity for chosen application (we evaluated products specifying 5000 and 8000 mAh at 12 V) and capable of providing sufficient current (up to 1A). The UPS battery packs we evaluated weighed<0.5 Kg. Whilst these products had a nominal 12 V output, in use they delivered between 10.6 and 12.1 V.

Incubation chamber one of two sizes:

- A flight case or briefcase with dimensions: W380 × H145 × D400 mm

or

- Vacuum flask- consumer hot food container, we selected a mid-sized 800 ml product, but larger and smaller versions are widely available.

Heating system:

- Temperature controller STC – 1000 rated at 12 V for switching 12 V heater.
- V resistive heater element; capable of providing up to 10 W heat.
- Small fan 20 × 20 mm or 40 × 40 mm either 12 V or 5 V (widely sold for 3D printers or computer cooling)

The power output of the UPS should be carefully matched to the heater element to avoid drawing higher currents than the battery is rated for.

Large MicroMI

For the large incubator version based on an easy to carry handheld flight case or briefcase, we constructed a simple rectangular frame from widely available aluminium extrusion (20 × 20 mm) plus a glass/acrylic sheet to build the main heated chamber which was fixed permanently to the bottom of the case with double-sided adhesive tape, providing enough space for incubating multiple standard samples. We found the flight case was quite heavy (2.6 kg for case alone), so lighter boxes of similar size might be better suited to some applications where portability is more important than strength. A transparent plastic sheet was connected to the extrusion using adhesive tape hinges on top of the chamber as a lid. The case was filled with foam insulation to reduce heat loss. The chamber was big enough to accommodate petri dishes, dip slides, cell culture bottles and microwell plates. A resistive heater element was fitted on the glass floor of the chamber and a small fan used to achieve homogenous distribution of heat throughout the chamber (Fig. 2). To facilitate consistent photo imaging of samples

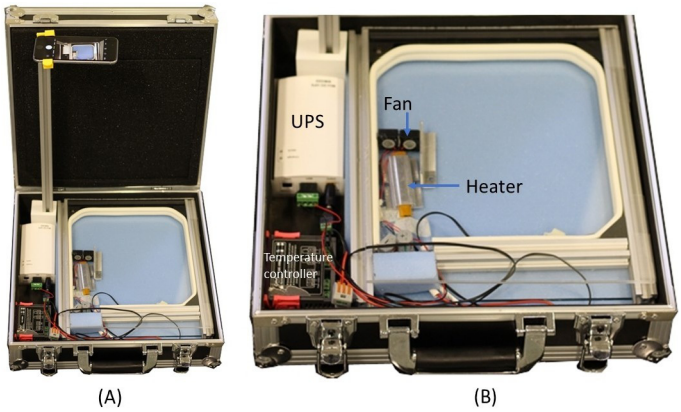

Fig. 2. Layout of large MicroMI based on flight case (A): Large MicroMI with cell phone mounted to take digital photographs and image microbiology samples during and after incubation without removing from incubator chamber. (B): Key components identified inside case including UPS power bank, temperature controller, and heated chamber layout including location of fans and resistive heating element.

Fig. 3. Small MicroMI based on food jar stainless steel vacuum flask (A): layout of MicroMI with temperature control and UPS power pack alongside (B): samples visible inside small MicroMI with upper lid removed (C): Small MicroMI in a small backpack for carrying and operation in the field.

during or after incubation the design included a cell phone dock, this was produced by 3D printing a bar holder and clip which fixed to the corner of the case above the transparent top of the incubator. In this way, samples can be imaged consistently without removing them from the incubator or even opening the incubator.

Small MicroMI

To avoid the need of carrying our larger version of MicroMI for field testing of a smaller number of samples, and to see if a better insulated incubator chamber could reduce the battery capacity requirement, we made a smaller version of MicroMI using a small vacuum flask in the form of a consumer stainless steel food flask intended to keep a small meal warm (800 ml). We 3D-printed a double-lid designed with a permanent outer lid ring that incorporates wiring to the heater, fan, and thermocouple that were all included inside the flask (Fig. 3), and a removable inner lid to close the chamber after samples were added. The power supply and thermostatic control module were kept externally, and the components transported together in a small backpack for portability.

Design files

Most components are freely available consumer products (listed below in bill of materials; BOM), however a small number of custom bespoke designs were used. A customised lid was designed and 3D printed for the vacuum flask. The smartphone camera mount requires two 3D printed parts. We provide here CAD designs (OpenSCAD) for modification to fit different vacuum flasks and/or smartphone sizes, plus STL mesh for direct 3D printing the exact parts used in building our own MicroMI. The full set of design files is also available at https://doi.org/10.5281/zenodo.5550785. All the design information including all essential information for procuring and making parts, and for assembly of the MicroMI is included in the tables below.

Design part name	File type	Location (all files are also available at https://doi.org/10.5281/zenodo.5550785)
Camera extrusion holder	STL	http://dx.https://doi.org/10.17632/mgkzyj8m9t.1
Camera extrusion holder	OpenSCAD	http://dx.https://doi.org/10.17632/t4dr6nnbn7.1
Phone holder to bolt on extrusion (Adapted from open source design: https://www.thingiverse.com/thing:11435)	STL	http://dx.https://doi.org/10.17632/pb9r7d2dgh.1; this STL has holder sized for Xperia L1 phone
Phone holder CAD design (Adapted from open source design: https://www.thingiverse.com/thing:11435)	OpenSCAD	http://dx.https://doi.org/10.17632/k7nt6g9c64.1
Lid for vacuum flask for small MicroMI	STL	http://dx.https://doi.org/10.17632/kr47gmm3m3.1
Lid for vacuum flask for small MicroMI	OpenSCAD	http://dx.https://doi.org/10.17632/dc7m6n9mm2.1

These four components were designed in OpenSCAD (or in the case of the phone holder, adapted from a previous open source design), and STL files were sliced using Cura software and transferred to the 3D printer to print the components using standard parameters:

- 3D Printer: Creality Ender 3 or Prusa I3 MK3
- PLA (Polylactic Acid): 1.75 mm
- Printing condition: 0.2 mm layer height, infill: 20%

Bill of materials

Component	Qty/unit	Cost per unit (GBP; purchase price in May 2020)		Source of material
		Large	Small	
Uninterruptable Power Supply (UPS)				NOTE: different capacity battery packs were compared; only 1 UPS needed per incubator
GM322 Mini UPS 7800MAH 12 V 2A – KTC5336FBA, 15.4 × 13.6 × 4.6 cm; 340 Grams)	1	26.99	26.99	https://www.amazon.co.uk/Docooler-Protection-Charger-Portable-Applications-White/dp/B07BF4SR6S
TalentCell Rechargeable 72 W 100WH 12 V/8300mAh 12 V/9V/5V DC Output Lithium-ion Battery Pack, YB1208300-USB – 13.7 × 3.9 × 7.9 cm; 500 Grams	1	56.99	56.99	https://www.amazon.co.uk/TalentCell-Rechargeable-11000mAh-20000mAh-Portable/dp/B06Y5G3C8Z
TalentCell Rechargeable 36 W 12 V/6000mAh 5 V/12000mAh DC Output Lithium-Ion Battery Pack for LED Strip, Tape Light, CCTV Camera and More, Black, YB1206000-USB, 12.95 × 2 .54 × 7.62 cm; 350 Grams	1	49.99	49.99	https://www.amazon.co.uk/TalentCell-Rechargeable-6000mAh-12000mAh-Lithium/dp/B0713T4XT9
Controller				
Aideepen STC-1000 DC 12 V-72 V LED Digital Temperature Controller Thermoregulator Thermostat with Heater And Cooler For Incubator, A7X13068,Accuracy: +/- 1° C (-50° C ~ 70° C).Temperature measuring range: −50° C ~ 99° C)	1	8.69	8.69	https://www.amazon.co.uk/Aideepen-Temperature-Controller-Thermoregulator-Thermostat/dp/B08DFPHZ3M
Heater				
Enclosure heating element 30 W, 80 °C, 12 → 24 V, 60x8.5x 35 mm Mfr. Part No.: FG14745.4	1	14.98		https://uk.rs-online.com/web/p/heating-elements/2995770/
Enclosure heating element 90 °C 12–30 V, 40x8.5x35mmMfr. Part No.:HPG-1/09-40X35-12–30	1		7.63	https://uk.rs-online.com/web/p/heating-elements/7256474/
Container				
Universal Flight Case – Medium - ACC-CASE-M, Dimensions: Inside (W × H × D): 350 × 115 × 350 mm, Outside (W × H × D): 380 × 145 × 400 mm, Weight: 2.66 kg	1	£39	–	https://cpc.farnell.com/pulse/acc-case-m/flightcase-universal-medium/dp/DP31699
Vacuum soup container Jar Lunch Box Food flask with handle 800 ml (18 × 12.2 × 11.8 cm; 650 Grams)	1	–	22	https://www.amazon.co.uk/ThermOwl-Stainless-Insulated-Leakproof-Container/dp/B07MDNJ6W4
Fan				
Axial Fan, Brushless Motor, Tubeaxial, Vapo, 5 V, DC, 20 mm, 10 mm, 1.5 cu.ft/min, 0.042 m^3/min	1	8.66	–	https://uk.farnell.com/multicomp/mc33873/dc-fan-axial-20 mm-5vdc-0-179a/dp/2395867

(*continued*)

Component	Qty/unit	Cost per unit (GBP; purchase price in May 2020)		Source of material
		Large	Small	
RS PRO, 5 V dc, DC Axial Fan, 40 × 40 × 10 mm, 11.9 m^3/h, 1.92 W	1	–	6.85	https://uk.rs-online.com/web/p/axial-fans/7897858/
Others				
V 2020 Black Aluminium Extrusion VSlot 6 Profile 20x20mm (2 × 1 m)	1	10	–	https://ooznest.co.uk/product/v-slot-linear-rail-20x20mm-cut-to-size/
50CM X 50CM X 5CM High Density Upholstery Firm Foam Rubber Sheet Cushion Replacement	1	10.25	–	https://www.amazon.co.uk/Density-Upholstery-Cushion-Replacement-Mattress/dp/B016ADSKJS
Double-side tape	<1m	Under £1	–	
90°Cast Corner	4	1.50	–	https://ooznest.co.uk/product/90-degree-cast-corner/
Silicone insulated wire 18 AWG Black and Red (approx. 1 m required)	1	6.54	6.54	https://www.amazon.co.uk/BNTECHGO-Silicone-Flexible-Strands-Stranded/dp/B01708AYYQ
WAGO Lever connectors 2-way (Part 222–412) and 3-way (Part 222–413)	1	2.70 plus 2.40 (10 per pack)	2.70 plus 2.40 (10 per pack)	https://uk.rs-online.com/web/p/standard-terminal-blocks/7581650/ https://uk.rs-online.com/web/p/standard-terminal-blocks/4751437/
PLA filament for 3D printing (white)	15 m 1.75 mm filament	under £5	–	https://ooznest.co.uk/product/pla-3d-printer-filament-1–75 mm/
		Total		
With *GM322 Mini UPS*		**£170**		
With *TalentCell 12 V/8300mAh*		**£187**		
With *TalentCell 12 V/6000mAh*			**£125**	

The total cost for each version (large vs small) of MicroMI depends on the capacity of UPS selected, as this was the most expensive single component. Two different types of UPS manufacturers were tested in the larger version of MicroMI and therefore, the total cost of building large MicroMI ranged from £170 to £185. For the smaller version of MicroMI only the 6000mAh TalentCell UPS was tested, so the total cost was £120. However, the price, availability and performance of these products varies considerably, and selection of different UPS should be made based on local availability and budget, and will be influenced by the duration the incubator will need to be used without any external power supply. As the power consumption varies depending on the ambient external temperature, careful consideration of battery capacity is required depending on the application and environment.

Build instructions

Large MicroMI

The following steps provide a step-by-step guide for building and assembling the larger version of MicroMI (Fig. 4):

1. Cut a hole (3.6 × 7.6 cm to fit dimensions of temperature controller) in the sidewall of the flight case and mount the temperature controller.
2. Cut the Aluminium Extrusion Slot Profiles into four pieces with the appropriate size to fit flight case interior.
3. Connect them together with nut and screw bolts using angle brackets.
4. Fix onto glass side and adjoin to the underneath of suitcase with double-sided adhesive tape.
5. Follow the instructions supplied with digital temperature controller product, connect positive and negative charge to 12 V power supply from the UPS, and to the heater, fan, and temperature sensor. Position these elements on the glass bottom plate to ensure fan distributes heat from the resistive heating element, and to ensure the temperature sensor is positioned near to the sample location.

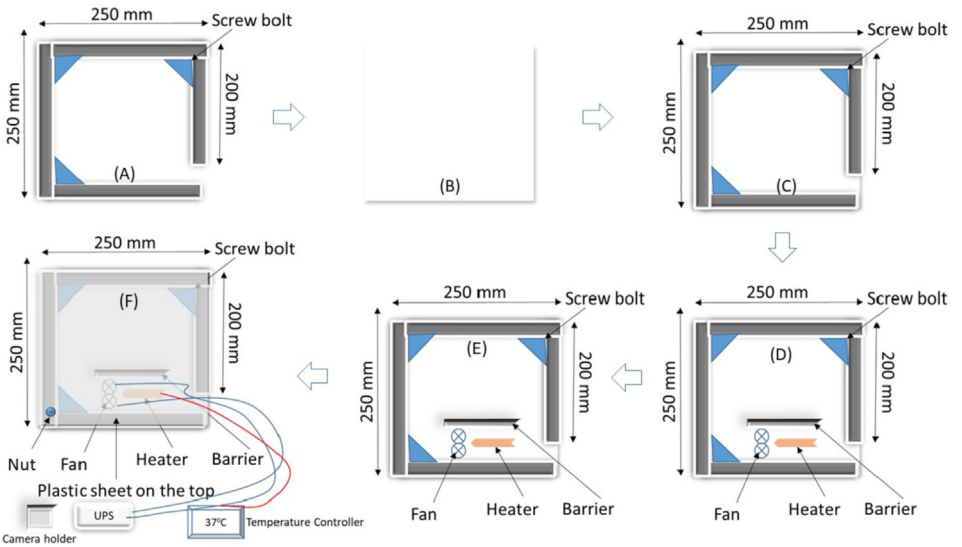

Fig. 4. Essential step to construction Heat – chamber for large MicroMI (A): Create the frame for the chamber using angle brackets bolted onto v-slot extrusion. (B): Glass plate fixed in the bottom of the flight case. (C) Fix chamber onto the glass plate using double-sided tape. (D): Fix fan and heater onto glass bottom. (E): Put the plastic sheet on the top hinged in place with tape. (F): Connect to battery and temperature controller. Then, fix UPS and camera holder into the case.

6. Put the plastic sheet (W308 × H145mm) on the top of frame, as a lid and fix with two layers of adhesive tape to hinge one edge. To secure the lid when closed, adhesive magnets can be fixed to the opposite corners to the hinge attached to the extrusion frame.
7. Use insulation foam (W308 × H145mm × D50mm or as appropriate to fit in chosen case) fixed into the bottom of the suitcase with double tape. This helps to maintain temperature inside and reduces heat loss, extending the time without external power.
8. Program the temperature controller to 37 °C as instructed by the manufacturer.
9. Put the UPS inside the flight case positioned in a convenient space to allow easy connection to an external power supply when available. We cut a rectangular hole in the case so the temperature screen and controls were visible outside, but this is optional.
10. Put the camera holder in the corner of suitcase at a location suitable for holding digital smartphone camera to image the samples within the incubator. The bar holding the camera clip is removable and can be stored inside the suitcase when not in use.
11. When capturing the photo, the phone holder is fixed on the Aluminium Extrusion using T-slot nuts. The bar is placed in the holder inside the case, then place smartphone into the holder clip and take the photo. The incubator lid is transparent. After finishing, cell phone and camera holder must be removed to close the lid of the case for transportation and to maintain temperature.

Small MicroMI

The follow step will help to summarize all essential steps (Fig. 5):

1. Put the vacuum custom 3D printed lid on the top of flask.
2. Fix the heater on the bottom side edge inside the flask.
3. Fix fan at the edge of the wall towards the bottom of the flask.
4. Follow the manufacturer instructions for the temperature controller to connect power, temperature probe, and heater.
5. Leave the temperature sensor inside the flask.

Operation instructions

These large MicroMI and small MicroMI are easy to use in many microbiological applications for agar petri dish, 96 well – plate, and dip-slides. This mobile incubator can set up range temperatures from below 37 °C to 42 °C for microbiota detec-

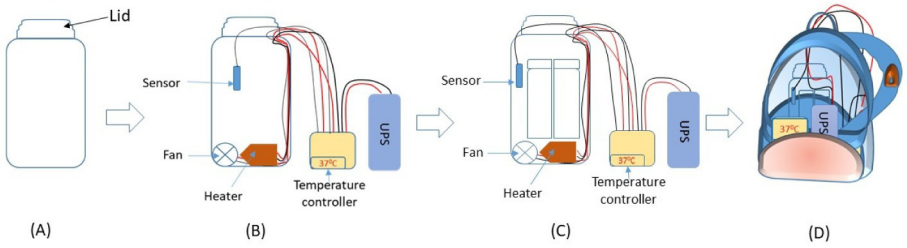

Fig. 5. Essential step to construct small MicroMI – vacuum flask (A): 3D-print a two-parts lid for the flask , (B): Add heater and fan to the bottom of flask which is connected to the temperature and UPS as introduction of temperature controller, with wiring routed through channel in lower lid. (C): Put samples such as frame dip slide, slant cultures, petri dish inside and add upper lid. (D): Small MicroMI can put inside the backpack to carry without interrupting operation.

tion, depending on the culture requirements. The device has no active cooling so cannot maintain temperatures below ambient without further modification. User instruction for operation is simple:

1. Carry this mobile incubator to the field location, temporary testing site, or anywhere required to perform the test.

2. Turn the MicroMI on at least 20–30 min before placing the samples inside the chamber so that target incubation temperature (37 °C) is achieved, based on large or small MicroMI respectively. Open the suitcase and lid of chamber to add samples.

3. As long as UPS battery is charged, incubator will operate without external power. However, when available, UPS unit should be plugged in allowing direct power by external source alongside battery charging.

This mobile device can be applied for biochemical testing of strains or used to culture and observe the bacteria present from samples collected from field sites, environmental or agricultural testing or clinical patient samples. In case of dip-slides which are simple, ready-to-use solid media for isolation and identification of many pathogens, just directly dip the dip-slides into samples and place into large or small MicroMI.

4. Appropriate safety equipment such as gloves, and good hand hygiene with detergent or antimicrobial such as alcohol is recommended prior to operation to maintain sterility and avoid contamination, and after handling samples. If handling clinical or potentially pathogenic samples, all essential microbiological safety procedures must be followed.

5. Close the large or small MicroMI and bring it back to local laboratory or reference laboratory.

6. The operating time without external power will depend on the ambient temperature outside; we evaluated these with an ambient temperature of approximately 18–20 °C (during UK winter) and 22–27 °C (during UK summer) and found temperature could be maintained from between 3 and > 24 h depending on the size and UPS capacity (Table 1). The smallest capacity UPS in the large MicroMI with under 20 °C ambient temperature only lasted ∼ 3 h, in contrast the mid-sized 6000 mAh TalentCell UPS pack maintained the Small MicroMI for 24 h.

The time maintained at 37 °C under battery power depended on the capacity of the UPS, with temperature stability and duration discussed in more detail below in section 7.

To validate the MicroMI we compared the results of a wide range of example microbiological assays including biochemical identification tests, agar petri dish colony culture, and smaller samples including slant tube and dip-slides (Figs. 6-9). We cultured three lab reference strains representing major gram-negative and gram-positive pathogens, *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 12600, plus *Klebsiella pneumoniae* ATCC 13883. In addition, 3D printed dip slides were used to

Table 1
Comparison of large and small MicroMI incubators.

	Large MicroMI	Small MicroMI
Time to reach 37 °C	35–40 mins	15–25 mins
Duration of power supply by UPS without any external power supply		
Power Bank Portable Power for 12 V (GM322 Mini UPS)	3–3.5 h with 15–20 °C ambient temperature	Not tested
TalentCell Rechargeable 36 W 12 V/6000mAh	Not tested	Over 24 h with 20–25 °C ambient temperature
TalentCell Rechargeable 72 W 100WH 12 V/8300mAh	8–9 h with 15–20 °C ambient temperature	Not tested
Time maintaining 37 °C after empty battery	1 h	2 h
Amplitude of temperature fluctuation	±0.5–1 °C	±0.5–1 °C
Application	Biochemical test, normal size petri dish, slant tube, frame dip slides.	Mini petri dish, slant tube, frame dip slides.

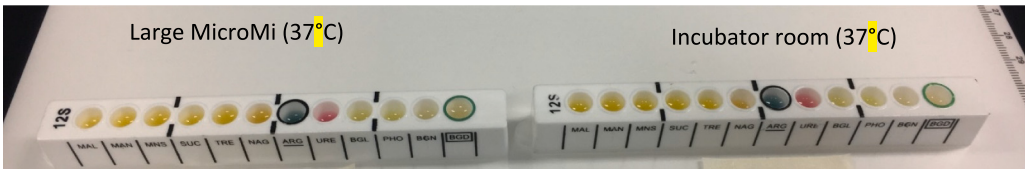

Fig. 6. Biochemical test performed in MicroMI alongside replicate in laboratory incubator room.

Fig. 7. Bacterial identification by colony growth on MacConkey's agar in small petri dishes performed using the MicroMI. The three reference strains were streaked onto three segments, and a fourth segment was negative control.

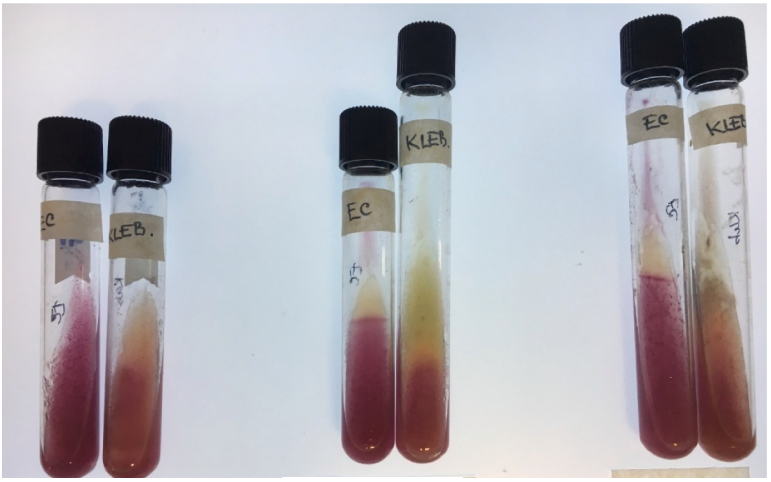

Fig. 8. Comparing bacterial growth in the MicroMI with conventional incubator– *E. coli* and *Klebsiella* on agar slant tubes of MacConkey agar.

detect bacteria in milk samples collected from cows with mastitis. All the results showed equivalent findings in terms of bacterial growth or biochemical test results observed using large and small MicroMI (both flight case and vacuum flask) to identical samples incubated in parallel in a conventional laboratory incubator room at the same culture temperature of 37 °C (Figs. 6-9) (Table 2).

The Microbact (Thermo Fisher Scientific Oxoid, Basingstoke UK) biochemical test panel for identification of different staphylococcus species was performed according to manufacturer's instruction, and with duplicate samples incubated either in the large MicroMI or a conventional microbiological incubator room (Fig. 6). This comparison did not include the small

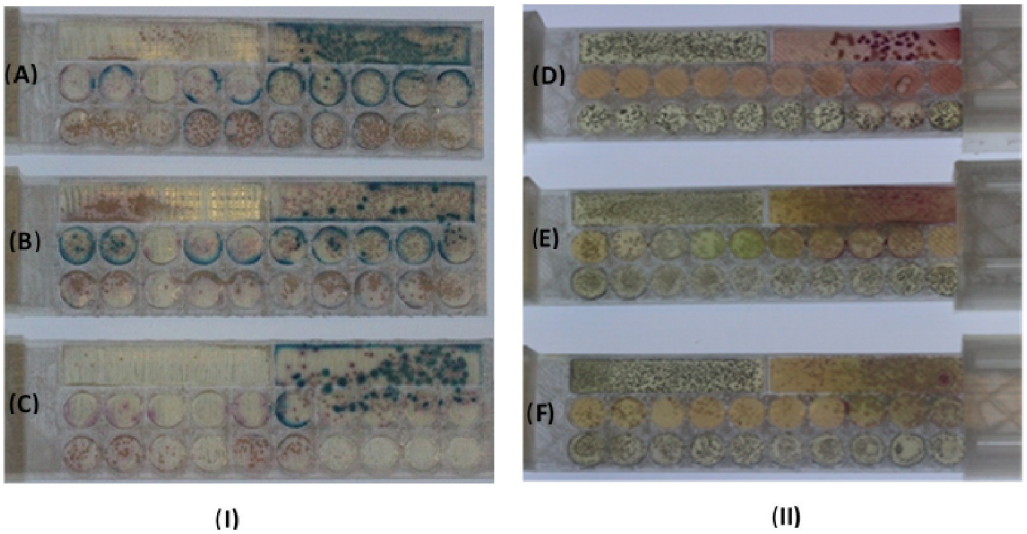

Fig. 9. Dip-slides constructed from 3D printed frames tested with mastitis milk samples (I), Frame dip slide on *E. coli* and *Staphylococcus aureus* ATCC strains (II). (A) and (D) were incubated at large MicroMI, (B) and (E) were incubated in the small MicroMI, (C) and (F) were incubated in a normal microbiological laboratory incubator.

MicroMI because these tests are liquid and the small MicroMI does not have suitable sample space for liquid microwell strips.

Biological safety requires careful containment of samples, and a range of measures are likely to be needed- as with use of any microbiological incubator and associated work practices. The large MicroMI has a securely closed lid which ensure the incubator chamber cannot open during transportation, but within the chamber careful planning is needed to ensure liquids or condensation cannot leak and microbially contaminate the inside of the incubator. We found hook-and-loop straps were ideal for securing the additional 3D printed lid onto the small MicroMI but sample containment is still required to prevent spillage that could contaminate the internal incubation chamber. The screw-topped slant tubes are enclosed after sample addition. Petri dishes require further containment such as zip-lock bags or parafilm. We 3D printed individual cases for the custom dip-slide frames (manuscript submitted). Sufficient air must be included to supply oxygen for any tests where aerobic growth is expected, which can be challenging when trying to seal samples against leakage. Biochemical tests in microwells cannot be transported as they will spill, so the MicroMI needs to be stationary during these tests.

In addition to biological safety, electrical safety should be considered. We used substantial spring connectors for rapid build and modification. We found the relatively low power drawn during heating of a peak of 1A (larger capacity battery in large MicroMI) and significantly lower peak of 0.5A (smaller TalentCell battery in small MicroMI) did not ever lead to any battery heating, even when the incubator heater chamber was heating up from ambient (i.e. heating element constantly on). As lithium batteries can potentially overheat and even catch fire if not used as directed, it remains important to use

Table 2
Comparison with the specified temperature control for commercially available microbiological incubator.

Manufacturer	Model	Temperature deviation	Preheat times (device empty, to 98% of working temperature)	Recovery times (device empty, door 30 s open, return to 98% of working temperature)	References
Manufacturers specification					
Thermo Scientific	IMC18	±1 °C at 37 °C	37 °C–15 min	37 °C–5 min	Thermo Scientific
Coleparmer	H2220-HE	±1.5 °C at 37 °C	Not specified	Not specified	https://www.coleparmer.co.uk/
Cultura[R]		±1 °C	Not specified	Not specified	https://echamicrobiology.com/
MicroMI comparison					
Our open hardware design - MicroMI		±0.5 °C at 37 °C	37 °C–30 to 45 min	37 °C–3 min	This paper- Fig. 10

these products safely and to ensure the UPS battery pack specification and any external 12v power supply used can provide the expected maximum current.

Validation and characterization

Alongside validation in use to perform microbiological tests, we performed technical performance, evaluating the stability and uniformity of temperature and the duration of operation.

We measured temperature at 5 points in the chamber (4 point at corner – 2 cm distance from the edge of chamber and 1 in the middle – 125 mm in the centre of the chamber), this temperature check was replicated at 3 different timepoints to assess range of temperature (Fig. 10). For small MicroMI, we measured two points: bottom and top – near the lid of flask, 30 mm long distance from the top. Similar temperature measurements were obtained with two different USB data loggers and the inbuilt temperature probe from the temperature controller, confirming the temperature controller was able to measure temperature accurately.

At each point, we fixed the sensor for 5 min, then measuring the temperature. The fluctuation of temperature was around 37 °C ± 0.3–0.5. All corner position varied around 37 °C with range of temperature of 0.01–0.03 °C. In the centre of the chamber, the temperature did not change substantially, the temperature stayed within ± 0.1 °C of the target temperatures and there was no significant difference in temperature between the sensor and data logger. For the small MicroMI, the temperature of bottom position was always higher than the top of flask. (Fig. 10)

Both the size of incubator and the ambient temperature outside are important parameters that influence the time to reach temperature for bacterial growth. For the large MicroMI, it took nearly 35 min to get 37 °C when the temperature outside was cooler (around 12–19 °C). The temperature only fluctuated between 0.3 and 0.5 °C around 37 °C, (Fig. 10), an acceptable variation in temperature that won't affect bacterial growth, and similar performance to commercial incubators (Table 2). The UPS maintained this temperature for 9 h in the large MicroMI before the largest capacity battery tested ran out and temperature fell, in contrast to the small MicroMI that maintained temperature for over 24 h without external power. The time depended on the battery capacity of the UPS, and the ambient temperature. For the small MicroMI, it took less time to reach 37 °C, only 16–23 min, with the fan ensuring rapid warm up and even temperature distribution (Fig. 10). We conclude the small MicroMI has superior insulation, as it stayed warm for far longer with the same capacity UPS and was faster to heat up, but it also had a smaller volume (thus fewer samples) that could also account for the lower power consumption and faster warmup. For the small MicroMI to achieve optimal incubation temperature for samples, the controller was set to 35 °C which ensured the homogenous heat inside did not exceed 37 °C, as we found with the data logger that the sample temperature was consistently 2 °C higher than the probe temperature. We conclude that minor calibration of the controller may be needed for each device.

To check the accuracy of the thermal controller temperature sensor probe, we used an additional data logger (Omega OM – EL USB – 2 – LCD). As the temperature logger recorded the same temperature as displayed on the temperature controller, we concluded the probe was calibrated accurately. We also used these USB data loggers to measure any difference in temperature at different points inside the incubator, and to see the rate of temperature drop after the UPS battery was depleted and the temperature controller switched off.

The large MicroMI lost around 5°after 1 h once the UPS battery was depleted, having stayed at target temperature for 4 h, using a smaller capacity UPS product. For this reason, we added a larger capacity UPS battery product, which lasted significantly longer. Because the larger size, larger heater element, and less effective insulation leads to far faster battery consumption, the large design is better suited to situations where there is intermittent access to external power. For example, during the day alongside travel away from external power, samples can be added to the incubator and culture experiment started. As long as external power – or a second fully charged UPS battery – is available after 4–9 h powered by battery, a full 24 h culture incubation period can be maintained. For example, the MicroMI can be plugged in overnight. Fig. 10C illustrates practical use of UPS to operate the incubator from 11:30am to 8:30 pm, at which point it was plugged in. By the following morning, mains power had maintained temperature and at the same time fully charged the battery. Larger capacity batteries are also readily available for additional cost if longer running is needed without external power.

We observed in the UK's temperate climate some differences in duration of temperature maintained by the battery between winter and summer periods. Covid-19 pandemic restrictions limited lab access during parts of this study, and limited access to temperature-controlled laboratory meant we could only test duration of battery for fixed times. Longer runs were performed without samples at home, during the summer, where ambient temperatures varied from 20 to 27 °C. We expect that in warmer ambient temperatures closer to typical culture temperature of 37 °C, such as tropical areas including Vietnam, Thailand, and Malaysia the temperature inside will be more stable, the UPS battery will last longer and the chamber will take longer time to cool down. With the current design these incubators can only be used when the ambient temperature remains at or below 37 °C, as no active cooling is included. However, the simple inexpensive thermal controllers used are also available with additional switched cooling circuit, so that active battery-powered cooling could be added alongside heating, if required. For example, fans plus a water source could be used to drive evaporative cooling. This remains an important opportunity for future innovation. Alternatively, the incubator could be operated in an air-conditioned location to avoid overheating.

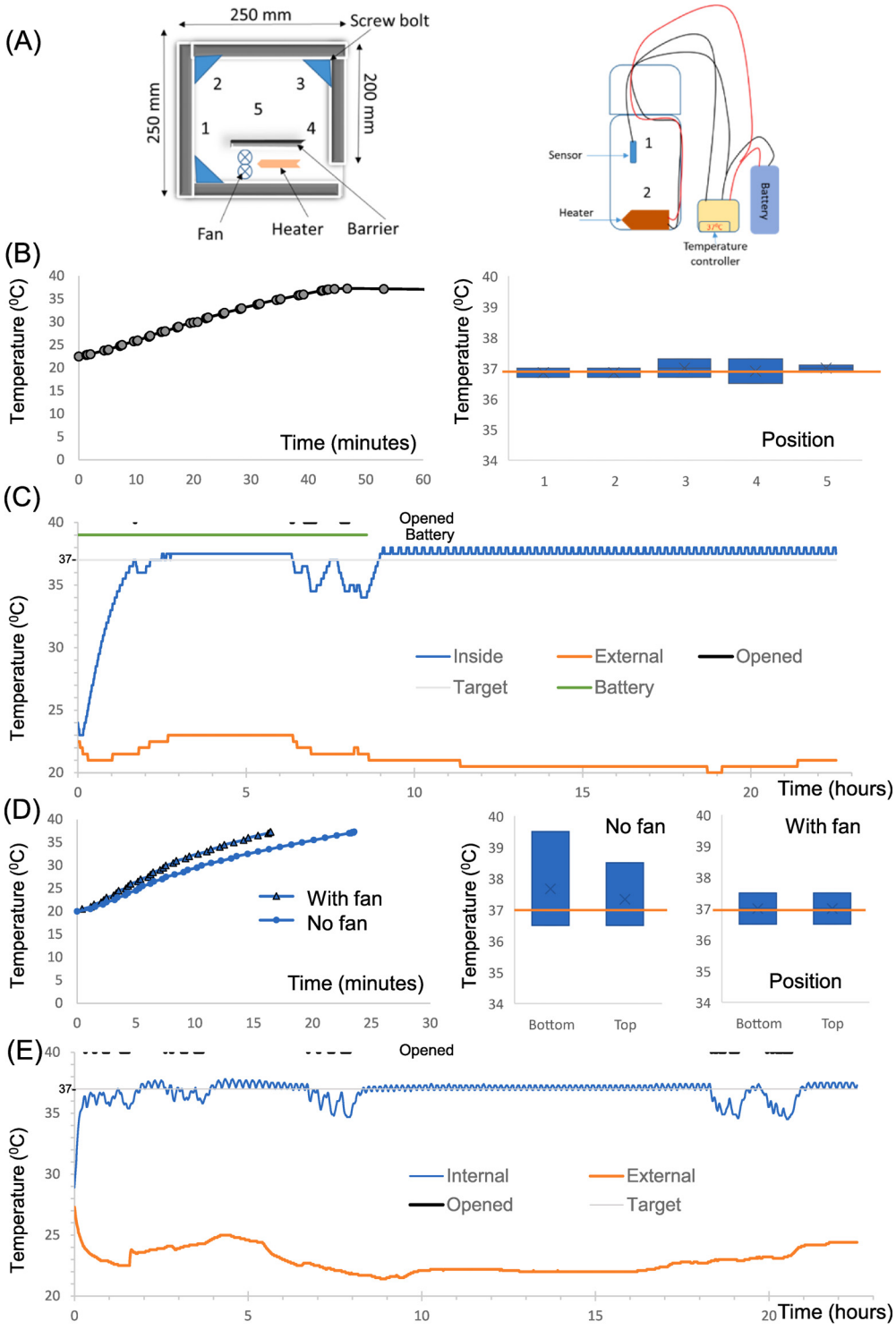

Fig. 10. The various temperature at different position. (A): Position of sensor in each MicroMI. (B): Time to reach 37 °C of large MicroMI and the homogenous temperature inside the chamber at different measure positions. (C): Datalogger temperature profile inside large MicroMI for 24 h operation, running for first 8.5 h until the UPS battery was depleted, and plugged into mains power thereafter (indicated by green line top). Black lines above indicate when incubator chamber was opened for increasing lengths of time, with respective dip and recovery in temperature. (D) Shows the increasing temperature to 37 °C from ambient, inside small MicroMI with or without fan (left), and shows how addition of fan leads to more uniform temperature than without (right). (E):Datalogger temperature profile inside small MicroMI for 24 h operation running on UPS battery alone. Black lines above indicate when incubator chamber was opened for increasing lengths of time to represent sample addition or removal, with respective dip and recovery in temperature. . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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.

Tai The Diep I completed a masters degree in 2005 from University of Natural Sciences in Vietnam. Now, I'm a microbiology researcher at Pasteur Institute of HCMC, Vietnam. In 2019, I received a fellowship from the University of Reading for my PhD with Dr. Edwards. My current project is developing new tools for detection of antimicrobial resitance in human health and agriculture. In addition, I'm also working on 3D printed Raspberry Pi microscopy for testing bacteria.

CHAPTER 5 - MOTILITY BASED AMR

Motility based AMR

This study described the application of 3D microscopy combined with a Raspberry Pi camera for early detection of antimicrobial resistance

Conference Abstract (**The 23rd International Conference on Miniaturized Systems for Chemistry and Life Sciences (µTAS 2019) -** Basel, SWITZERLAND

Tai The Diep, Alexander Daniel Edwards (2019). 3D printed Raspberry Pi Microscopy for low-cost Microfluidic bacterial motility analysis**. Poster presented.**

5.1.1. **Title**: 3D printed Raspberry Pi Microscopy for low-cost microfluidic bacterial motility analysis.

5.1.2. Where: The 23rd International Conference on Miniaturized Systems for Chemistry and Life Sciences (µTAS 2019) - Basel, SWITZERLAND

5.1.3. Full poster

School of Pharmacy

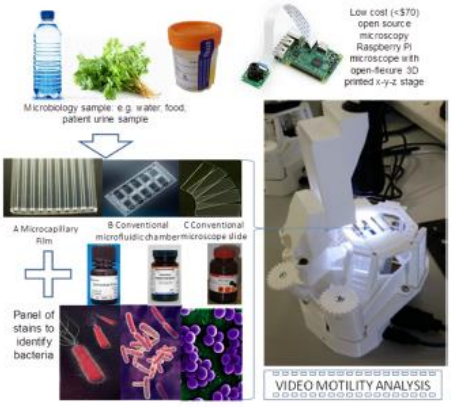
University of Reading

3D PRINTED RASPBERRY PI MICROSCOPY FOR LOW COST MICROFLUIDIC BACTERIAL MOTILITY ANALYSIS

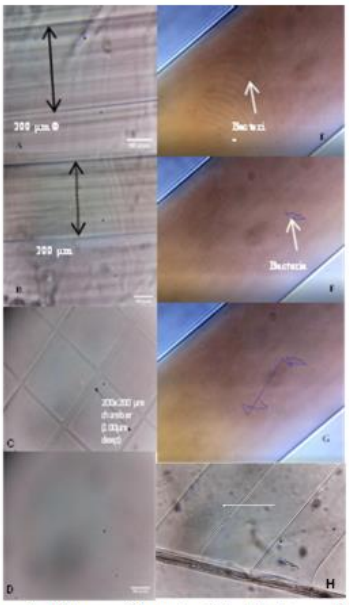
Tai The Diep and Alexander Daniel Edwards

ABSTRACT

We combined 3D printed Rasberry Pi microscopy with microfluidic sample chamber for rapid and simple detection of bacterial motility. From this system, image and video were taken and analysised rapidly.



Combining 3D Printed Raspberry Pi microscope with microfluidic sample preparation for rapid bacterial motility detection



Example still images of bacteria within A, B microcapillary film, C microfluidic hemocytometer, and D conventional slide and coverslip either unstained (A, C, D) or stained with phenol red (B). E,F,G: stills from video analysis of bacterial motion by Image J particle tracking. (H). Bacteria come to cluster

	Microbe detected			Motility detected		
		Spiked:			Spiked:	
	-	High	Low	-	High	Low
Human urine (sterilised)	NA	+++	-	NA	-/+	-
Bottle mineral water	+	+++	+	+	+++	+
Coriander/ Cinlantro	+	+++	+	+	+++	+

Direct detection of bacterial motility

CONCLUSION

The low cost and portability of all components (total materials cost of ~$60, and <200x200mm footprint excluding computer keyboard/mouse/monitor) make this technology accessible for teaching, research, field testing such as investigating outbreaks, as well as diagnostic microbiology in hospital and community settings.

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5.2. Full paper

Tai The Diep, Samuel Bizley, Alexander Daniel Edwards (2022). **Integrating 3D printed microscopy with advanced MCF technology to detect bacterial motility: an approach to rapid detection of antimicrobial resistance.** This work is planned to publish in Micromachines Journal with the tile

The extent of Contribution to research

In this work, I:

1. Printed a 3D microscope
2. Designed the experiments
3. Performed the test
4. Collected and analysed the data
5. Drafted the manuscript

Integrating 3D printed microscopy with advanced MCF technology to detect bacterial motility: an approach to rapid detection of antimicrobial resistance

Diep The Tai, Sam Bizley, Al. Edward

Department of Pharmacy, Reading School of Pharmacy, University of Reading, UK

Abstract

Early detection of antimicrobial resistance (AMR) is attracted much concern in public health. Motility-based AMR, the phenomenon of motile turn non-motile of bacteria when interacting with antibiotics, can early detect antibiotic susceptibility test. Based on the current design of 3D printed Openflexture microscopy, combined with Raspberry Pi camera model B and using Micro capillary Film (MCF) contained antibiotics in the semi-solid agar (0.4%), a new system was built up for the detection of resistant bacteria. This system was tested for *E.coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145, and strains isolated from mastitis cow milk samples with the panel of antibiotics including ampicillin, amikacin, cefotaxime, gentamycin, and amoxicillin. All photo and video recordings were captured by Python programming language and showed up on the computer screen. This study showed that bacterial motility can be seen in the microcapillaries by using 3D printed Openflexture microscopy, combined with Raspberry Pi camera model B. In addition, antibiotic susceptibility could be found around 5 to 10 minutes with this system based on the phenomenon of motile turned into non-motile bacteria in semi-solid agar. Using this system is a potential tool for enhancing the flexibility of microbiological laboratory, especially in the direct detection of bacteria, and resistant

strains on the sample. The fact that expanding this new technique can contribute to teaching, doing research, and investigating outbreaks at sites, especially in hospitals and communities.

KEYWORDS: bacterial motility, susceptibility test, 3D printed Openflexture microscopy, Raspberry Pi, microcapillaries.

1. INTRODUCTION

Bacterial motility is one of differentiating characterization species as well as the mechanism of approaching the nutrient source for their living. Motility has been widely acknowledged as a virulence factor in some pathogenic bacteria and has played a critical role in the formation of biofilm, which increases bacterial tolerance to antibiotics (1, 2). For the last several decades, different methods have been developed to differentiate and classify bacteria based on their motility. There are four kinds of different movement of bacteria as darting motility is presented by *Vibrio* sp, active motility: *Salmonella*, sluggish motility: *Bacillus* and *Clostridia* genus, thumbing motility: *Listeria.* The motility test has just not only one characterized help to identification and differentiation of each bacteria family, but understanding the bacterial movement informed us about the microscopic world as well as is essential in physic research and chemical gradients which is crucial for health, environment and agricultural (3, 4). However, many factors such as temperature, pH, chemistry (3, 5), moisture, and the viscosity substrate (6) influenced bacterial motility which is one important factor to affects biofilm formation. Biofilm formation is one of the mechanisms to protect bacteria against antibiotics and turned into resistance (7).

Initial developments of bacterial motility tests included the use of culture medium and naked-eye observation such as in the Craigie, J. (1993) tube method, which was then advanced by the inclusion of microscopic examination (e.g. the Wet mount method) (8). Furthermore, there some reports had applied new techniques to enhance the test such as the vital staining method with carbon fuchsin, safranin, and methyl blue or a novel single-tube agar-based technique for motility enhancement of *E.coli* O157:H7, and microscopy-based assay for quantifying in situ growth had been developed (9, 10).

Recently, microfluidics-based diagnostic technology has improved the healthcare systems by allowing the development of low-cost, rapid point-of-care diagnostic devices (11). Interestingly, microfluidics had been applied for rapid detection of susceptibility tests, one of the tackled problem issues on the global such as producing microchips for detection of MIC. But this required many steps to produce the chips and needs more staff training and still performed in the laboratory. Combining the Micro capillary Film (MCF) has been produced by Alex Edwards, at Reading University, used for microbiology, a promising alternative material for bacterial motility assay with Raspberry Pi, a UK-based charity, made it possible for non-tech people to use the power of computing and digital making which can be observing the bacterial morphology and essential tool for teaching, scientific analysis anywhere (12), motility testing can be performed in fields and contribute to reduce diagnostic time and get the right treatment for patients. Therefore, the study aimed to observe the bacterial movement in microcapillaries containing antibiotics loaded in semi-

solid agar (0.4%), then applied this system to early detection of bacterial antibiotic resistance through the phenomenon of turned bacterial motile to non-motile.

2. MATERIAL AND METHODS

2.1. Build up of new testing system including 3D printed microscopy and Raspberry Pi camera

Via the GitHub of Richard Bowman called open flexure microscope project - https://github.com/rwb27/openflexure_microscope, most parts of microscopy had been downloaded and printed out for our study. Then, all parts were printed by a Prussa printer, a product of the Prussa company, and got assembled through instructions. This printer was also used to print the holder of MCF for our method. The design can be found in my Mendeley data (DOI: 10.17632/79rd727xs3.2 and DOI: 10.17632/2nc6jhmhfw.2). Raspberry Pi (RPi) 3 Model B which connects to mouse and keyboard, computer screen had been set up. Camera 2.0 version was used to take the photo or video recording. Python code scripts were used to control the time to take the image and record the video which set up the resolution (1920, 1080) and captured 15 frames in each shot.

2.2. Preparation of bacterial strains, dye, and prepare spike samples.

The *E. coli* ATCC 25922 (EC), and *P. aueruginosa* ATCC 10145 (PSA) strains were subcultured on Luria Bertani (LB) broth (ThermoFisher, UK), overnight and were serially diluted (×1/10) before testing for motility. Next, they also used to spike on cilantro, urine

samples, and bottle mineral water to check whether we can detect directly bacteria on samples.

E.coli, and Pseudomonas aeruginosa have were isolated from bovine mastitis milk samples which knew the MIC value was applied to test for bacterial motility (Table 1). The known MIC helped to confirm one more time the value of MIC in the new system. Those strains were inoculated into MH broth at 37^0C overnight, then took 10 µl for motility test as well as a susceptibility test.

Table 1: MIC breakpoints of mastitis strains

Testing strains - mastitis isolates	MIC of antibiotic (µg/ml)				
	AMP	AMO	AMI	CEFO	GEN
19.02 MRF (EC)		32	16		4
19.10 MRF (EC)	32	8		16	2
19.16 MRF (EC)		16		8	32
19.14 MRF (PSA)	32	32	32		
20. 20 MRF (PSA)		32			8
19.08 MRF (SA)	32		16	16	

2.3. Preparation of MCF for performing motility and determining antibiotic resistance.

MCF was manufactured by Lamina DielectricsLtd (Billlingshurst, West Sussexx, UK) (13) with two sizes of capillary: 500 µm and 200 µm was used for this study. To make sure inner microcapillaries are hydrophilic, polyvinyl alcohol (PVOH) 20 g/l was coated overnight and dried with a vacuum. This step is easy for loading the samples get in the

MCF. We simultaneously loaded semi - solid agar (0.4%) into MCF instead of PVOH coating.

In addition, to perform the inhibition of antibiotic bacterial movement, a range of antibiotic concentrations from 0.0125 μg/ml to 32 μg/ml was prepared in the Muller Hinton broth containing 0.4% agar, then this mixture was injected into each capillary. This MCF was used to screen bacterial antibiotic resistance. The interpretation result is based on bacterial motility. The susceptibility considered as bacteria was stopped moving after interacting with an antibiotic and resistance was bacteria kept moving after interacting with an antibiotic.

2.4. Comparison of bacterial movement in MCF and well.

To confirm our record on live observation, the semi-solid agar method was applied to compare with our design system - combining MCF with frame holder and 3D printed microscopy. To prepare semi-solid agar, we use Muller Minton 0.4% contained antibiotic with dilution range from 0.0125 to 32 μg/ml in each ELISA plate well. The total volume of semi-solid agar was 200 μl contained antibiotic and triphenyl tetrazolium chloride (TTC) dye (1%) (Sigma, Gillingham, UK). Then, 10 μl of liquid bacterial culture was stabbed into the surface of the agar and incubated at 37^0C during 16 - 18 hours. Bacterial visible stab line and cloudiness were recorded as positive - bacterial motile which is considered resistance. In contrast, the visible stab line with clear agar was recorded as negative-bacterial non - motile which is considered susceptibility. In this experiment, ampicillin,

gentamycin, cefotaxime, amoxicillin, and amikacin were applied to screen the bacterial resistance to the antibiotic.

2.5. Spike bacteria into food samples and test their motility

All reference diluted strains (10-fold dilution) were spiked on cilantro and bottled water (bought from a local retail store). Liquid human urine (Biorad, Watford, UK) was used as a negative control. The motility of bacteria in all spiked samples was tested using MCF. The range of bacterial concentration was 10^1CFU/ml to 10^5 CFU/ml was used for the test.

2.6. Observer and track the bacterial motility

To the observer, the motility, bacterial solution, spiked samples, or milk samples were taken at 10 μl for letting into the PBS buffer covered MCF contained semi-solid agar (0.4%). The 3D printed microscopy was applied to observe the motility or non - motile of bacteria.

Video record from Raspberry Pi and digital camera of microscopy was transferred into ImageJ to analyse the motility tracking.

3. **RESULTS**

3.1 Observe bacterial movement inside MCF combined with 3D printed microscopy

The bacterial movement in the MCF can be seen as clearly as on the glass slide. Our proof of concept using MCF instead of a glass slide or haemocytometer as a carrier for observation allowed us to see the bacterial motility as well as their susceptibility to the antibiotic. Through this system, there were two options: using different antibiotics

combined with a negative control without antibiotics in one strip of MCF or may choose one antibiotic with a range of diluted antibiotic concentrations in one strip of MCF (Figure 1A). In addition, when seeing bacterial motility without semi-solid agar (0.4%) and buffer, the quality of the video or image was blurry. Therefore, coated semi-solid agar inside MCF and put under PBS buffer increased dramatically the quality of video or image of bacterial motility (Figure 1B).

Moreover, it was easy to handle with 3D printed microscopy combined with computer screen and captured the video or photo with Raspberry Pi. The 3D printed microscopy has two small gears to adjust the left and right position of samples, and one middle gear to focus on the samples. Literally, the video or image was captured by camera 2.0 as camera as a cell phone connected to the control board of Raspberry Pi (Figure 1C). It was not difficult to transfer the video into ImageJ to further analysis. Then, particle tracking analysis by ImageJ was used to quantify motility.

Figure 1: The concept of combining 3D Printed Raspberry Pi microscope with microfluidic sample preparation for rapid bacterial antibiotic resistance detection (A: Concept of using MCF combined with an antibiotic, B: MCF under holder with buffer, C: Observed the bacterial motility by 3D printed Microscopy and MCF)

3.2. Direct detection of contaminated bacteria through their motility

The new system was found to be adequate for direct detection of bacterial movement from typical microbial safety testing samples such as vegetable washings and bottled water, either spiked with known concentrations of microbes or when real samples were directly

tested (low-level contamination). Likewise, bacteria (10^1 to 10^5 CFU/ml) were visibly spiked into sterilized control human urine, although undiluted human urine did reduce detectable motility (Table 2). From spiked samples, the bacterial motility was seen with MCF coated with semi -solid agar (0.4%). When using liquid samples, the PBS buffer didn't need to use to increase the resolution of photos or videos. The phenomenon of movement was clearly seen on computer screen within MCF.

Table 2: Direct detection of bacterial motility

	Microbe detected			Motility detected		
	Spiked:			Spiked:		
	-	High	Low	-	High	Low
Human urine (sterilized)	NA	+++	-	NA	-/+	-
Bottle mineral water	+	+++	+	+	+++	+
Coriander/ Cilantro	+	+++	+	+	+++	+

3.3. Early detection of motility based antimicrobial resistance (AMR)

After interacting with antibiotics, bacterial motility changed into non-motile. The changing time depended on the type of antibiotic and trains. The higher concentration of antibiotics, the quicker bacterial motility was inhibited. When observing the motility of *Pseudomonas* at various concentration of antibiotic, the bacterial death line was found. In

addition, the distance of moving was shorter at high concentration of antibiotic. This phenomenon has just seen on Pseudomonas only, unseen on *E.coli* (Figure 2).

Figure 2: Impact of antibiotics on bacterial motility. *Pseudomonas aeruginosa* (PSA) (A: negative control, B: 1mg/ml of Gentamycin inhibited bacteria, C: 2mg/ml of Gentamycin, D: 4 mg/ml of Gentamycin, B - D: bacterial line dead after inhibited by antibiotic) and *E.coli* (E: negative control-F: bacterial stop moving around at 1mg/ml of Gentamycin)

Furthermore, the motility-based AMR methods have the agreement results with semi-solid agar methods. Figure 3 showed that Pseudomonas couldn't growth at high concentration of Gentamycin. In the low concentration, bacteria grew and spread out the surface of media and from the inoculate points (Figure 3). This double-checked with semi-solid agar to comparison the results observed on microscopy.

Figure 3: *Pseudomonas* stop moving at various concentrations of Gentamycin on semi-solid agar (0.4%) in well

In general, most bacteria-referent strains isolated from mastitis milk samples became non-motile from 5 minutes to 10 minutes. At the high concentration of antibiotics (gentamycin, amikacin, cefotaxime) from 16 to 32 μg/ml, the bacteria changed from motile into non-motile just for 1 minute to 2 minutes. On the Ampicillin, bacteria kept moving even at high concentrations (32 μg/ml). Amoxicillin quickly affected bacterial movement; it took around 5 minutes to turn bacteria into non-motile (Figure 4).

Figure 4: Time taken for E.coli change from motile to non-motile in varying concentrations of amoxicillin.

All strains including referent strains or mastitis isolates (3 strains of *E.coli* and 02 strains of *Pseudomonas*, 01 *Staphylococcus* strain) elucidated the changing time between motile to non-motile that depended on the type of bacteria and antibiotic. However, non-motile was recorded around 5 - 10 minutes for high resistant strains. Most bacteria turned into non-motile at the inhibited concentration of antibiotics, there are still a few single bacteria that keep moving for a long time before stopping moving compared to the whole bacterial population. This phenomenon was also found the same when directly detecting bacterial resistance in samples. In real samples, there are always many more bacterial species instead of only one single species. Meanwhile, some will be stopped moving, others keep moving because of resistant strains. If the strains were resistant, kept swimming after 10 minutes, the susceptible strains stopped swimming at around 10 minutes or less than 10 minutes (Video 1 and video 2).

261121 PSA in MCF - NegCefoxitin.avi

Video 1: Bacterial motility without antibiotic - Negative control in MCF.

261121 PSA in MCF Cefoxitin 32.avi

Video 2: Bacterial non - motility in MCF.

4. DISCUSSION

This new system is dynamic - easy to set up anywhere, and could be flexibly used with any material such as slide, routine methods in the microbiological laboratory, a cheap material - MCF which simultaneously detect ten different concentrations of antibiotic. Interestingly, MCF is new material, not sharp, no harm to children - was able to use for teaching. Combining with 3D print, many labware tools for microbiology can be created, can be reused, and ready to use (14). In this study, a holder for MCF was produced to perform available routine work in the microbiology laboratories. Not only was a holder for MCF, but microscopy was also printed from 3D design which reduced the cost of investment compared to digital microscopy. Moreover, this assay can perform in the fields and take direct samples into MCF and detection of AMR. However, the resolution of the Raspberry Pi camera was affected by the type of samples, and their background color. Therefore, the dye should be carefully selected for use in a motility test to be able to see the shape of bacteria. In this study, TTC dye was good stains for bacterial motion. The resolution of the camera was another factor that affected the quality of video recording, increasing the resolution of the camera or using objectives with higher magnification will be a good way to amplify the size of bacteria. In addition, the natural color of samples contributed to the effect to the quality of the image such as milk being cloudy and dark white, and urine being dark brown so it was quite difficult to capture the photo or record the video. However, this didn't affect so much when seeing directly through the screen of the computer. Bacterial

movement and inhibition of antibiotics were still observed by eyes through computer screening.

This assay could be contributed to a new alternative approach to deal with microbial antibiotic resistance nowadays. In the microbiological laboratory, an antibiotic susceptibility test had been applied by a system of Phoenix (Becton Dickinson, USA), Vitek (BioMerieux, France), or disk diffusion test (Kirby Bauer), E-test, one commercial product from Oxoid, Liofilchem. However, laboratories need to invest large money for doing that. A simple way to check bacterial motion turn into death or an imperturbable situation. We can predict quickly the antimicrobial resistance, just 5 minutes or less than that. Parallelly, video recording from this system is also actively learning for the student and storage for doing research. Despite this early, our assay has just applied for motile bacteria, not for non-motile bacteria such as *Klebsiella,* some *Staphylococcus.* Controversially, bacteria didn't isolate in their niches, and still, bacterial evolution has always happened and leverage from other motile bacteria has been found and able to motile from non - motile have been reported such as *Klebsiella* (15), *Staphylococcus* (16). The bacterial motility has still unclear yet. Furthermore, our assay was applied directly from samples that contained many bacterial species, not only single bacteria, even non-motile bacteria in it. Therefore, the alarm signalling of resistant strains that occurred in the sample has still valuable to think about the way to give the right choose antibiotic and treatment to patients. Our assay has promised to directly and early detection of bacterial resistance in this rising awareness of public health on AMR.

Notably, observation of contaminated samples was directly performed, especially bottled water which is increasingly reported in contamination cases and transmitted in many countries where 30,000 people and children died every day from water-associated diseases, according to World Health Organization has reported. Many countries had found *Pseudomonas*, a potential hazard pathogenic to health, in drinking water in Brazil, Canada, France, Germany, Spain and United States, Nepal, Iran and Nigeria, Sri Lanka (17-20). Not only had bottled water got a serious problem, but vegetable was also another aspect of food poisoning related to bacteria on fresh salad, insufficient consumption of vegetable in global and Africa such as Cameroon (21). Detection at the right time will contribute to stopping the spreading of pathogenic agents transmission.

5. CONCLUSION

These initial tests indicate that 3D printed Raspberry Pi digital microscopy combined with microfluidic sample preparation has great potential for direct microbiological analysis of food, water and clinical samples, especially on antimicrobial resistance. The low cost and portability of all components (total materials cost of ~$60, and <200x200mm footprint excluding computer keyboard/mouse/monitor) make this technology accessible for teaching, research, field testing such as investigating outbreaks, as well as diagnostic microbiology in hospital and community settings.

AUTHOR CONTRIBUTIONS

Conceptualisation—DTT and AE. Methodology and design—DTT and AE. Investigation—DTT. Writing—original draft—DTT. Writing—review and editing— SB and AE.

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CONFLICTS OF INTEREST

None

DATA AVAILABILITY STATEMENT

All data was available in this manuscript and at Mendeley data at DOI: 10.17632/79rd727xs3.2 and DOI: 10.17632/2nc6jhmhfw.2.

ETHICS STATEMENT

None required

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Supplementary figure

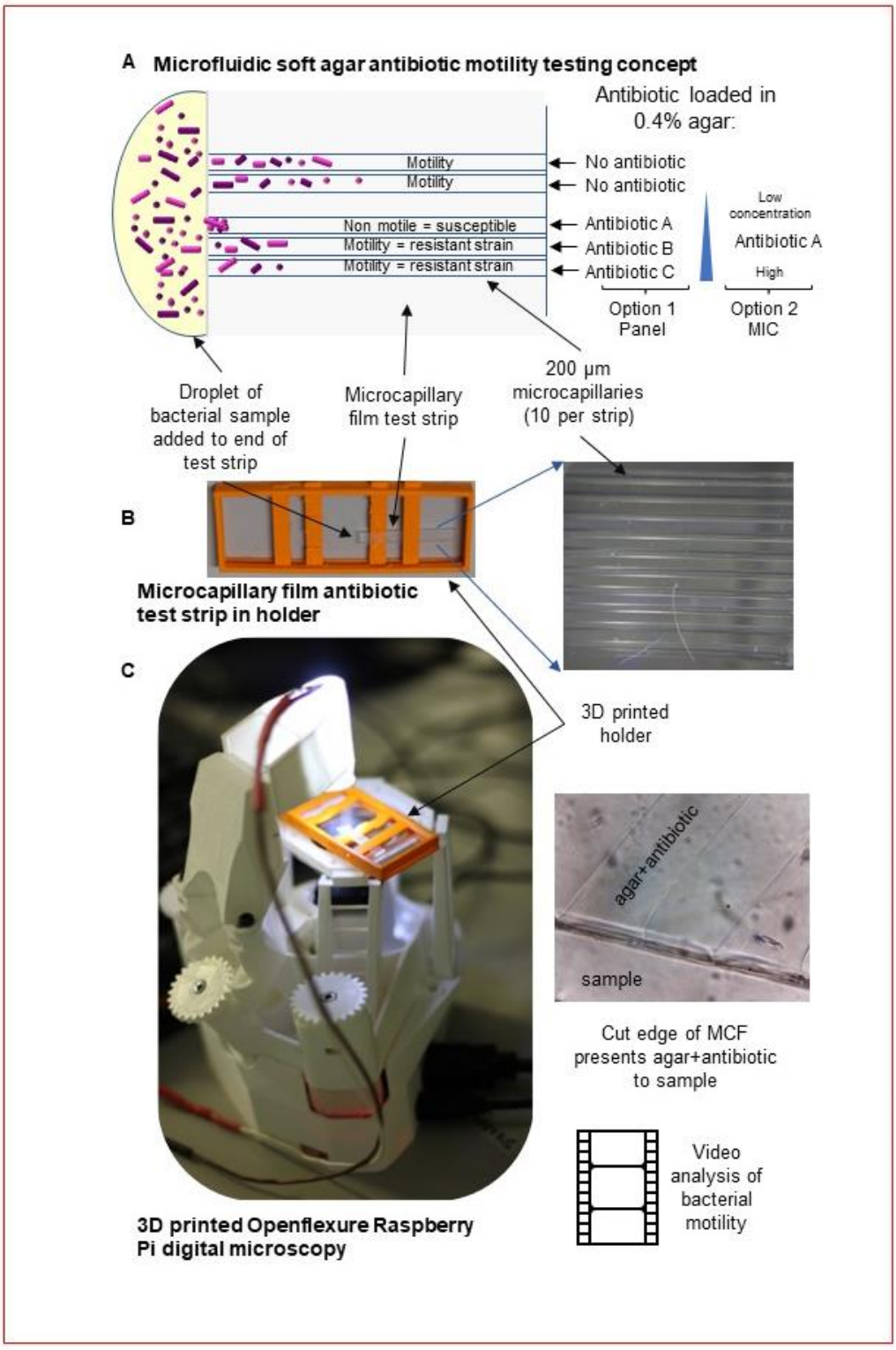


Figure 1: The concept of combining 3D Printed Raspberry Pi microscope with microfluidic sample preparation for rapid bacterial antibiotic resistance detection (A: Concept of using MCF combined with an antibiotic, B: MCF under holder with buffer, C: Observed the bacterial motility by 3D printed Microscopy and MCF)

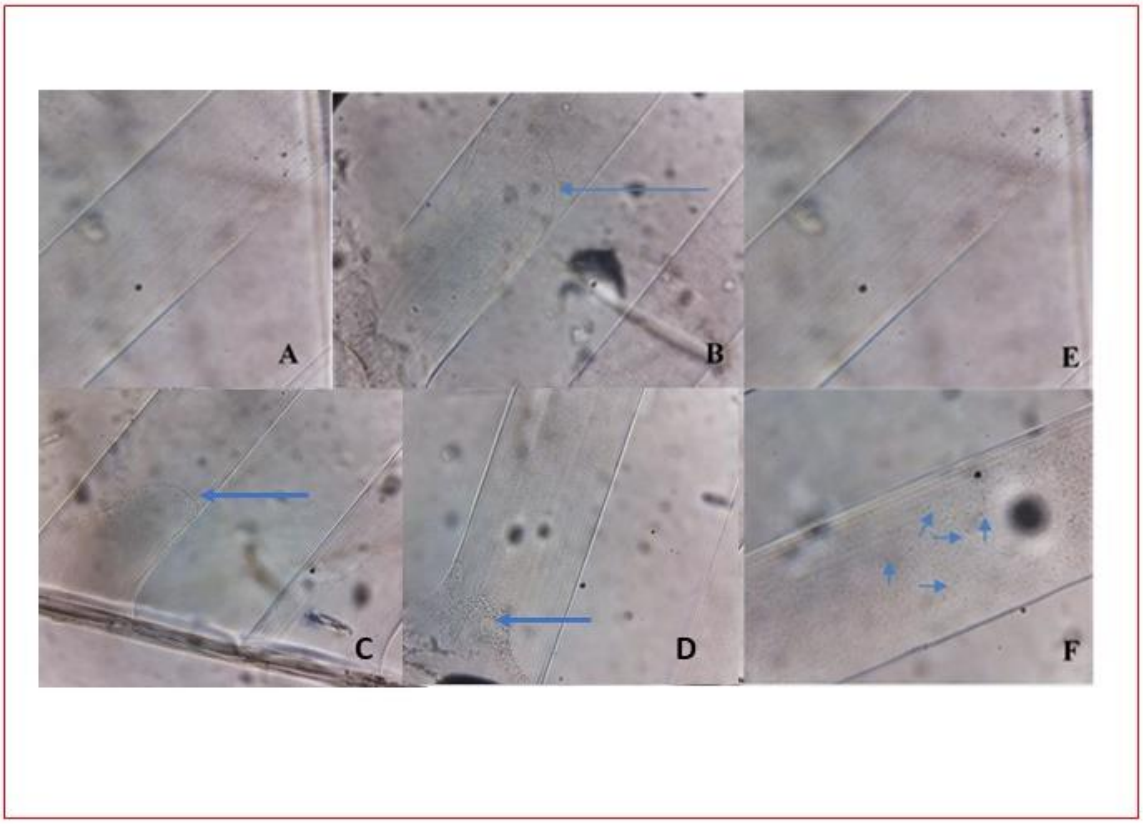


Figure 2: Impact of antibiotics on bacterial motility. *Pseudomonas aeruginosa* (PSA) (A: negative control, B: 1mg/ml of Gentamycin inhibited bacteria, C: 2mg/ml of Gentamycin, D: 4 mg/ml of Gentamycin, B - D: bacterial line dead after inhibited by antibiotic) and *E.coli* (E: negative control-F: bacterial stop moving around at 1mg/ml of Gentamycin)

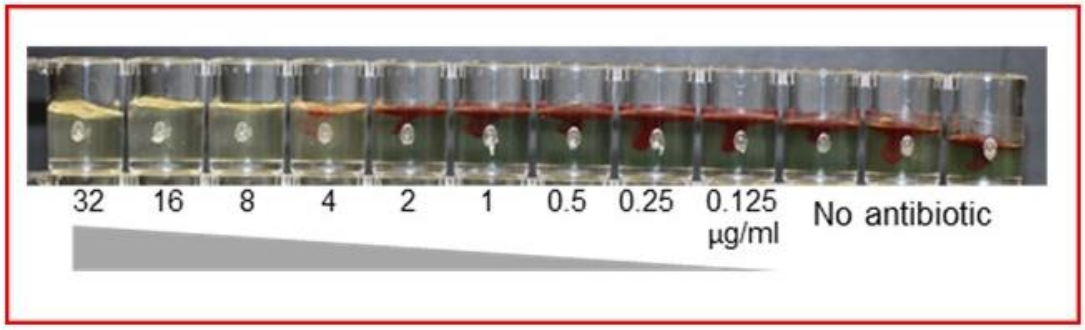


Figure 3: *Pseudomonas* stop moving at various concentrations of Gentamycin on semi-solid agar (0.4%) in well

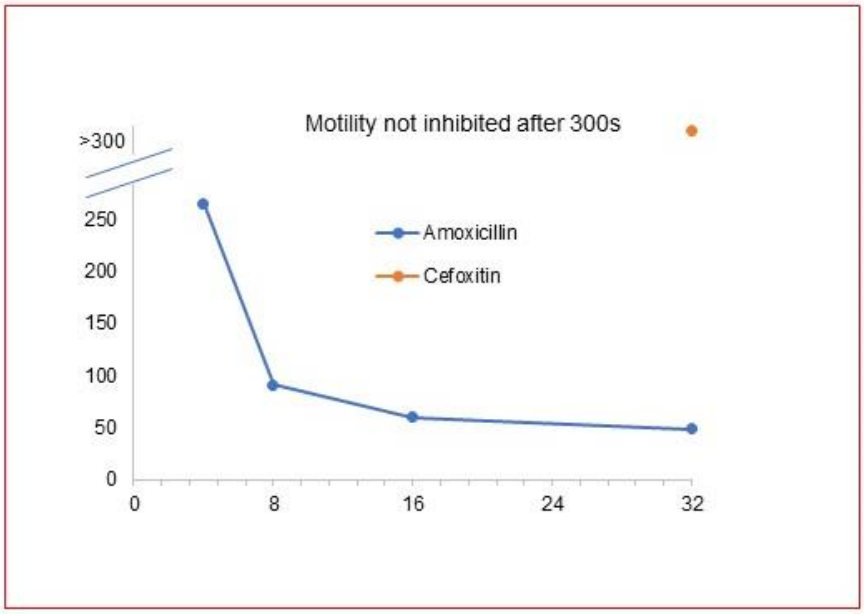


Figure 4: Time taken for E.coli change from motile to non-motile in varying concentrations of amoxicillin.

CHAPTER 6 - AUTOMATE TIME - LAPSE IMAGING

Automate time-lapse imaging

This study described the application of 3D architecture for laboratory robotic

Conference Abstract (54th US-Japan Cholera Conference in Osaka - 2019)

Diep The Tai, Sarah Needs, Alexander Daniel Edward (2019). Open-source hardware: Automated time-lapse for visualizing bacterial colony and sugar utilization with POLIR. **Poster presented.**

6.1.1. Title: Open Source Hardware: Automated time-lapse for visualising bacterial colony & sugar utilisation with POLIR

6.1.2. Where: 54th US -Japan Cholera Conference in Osaka - 2019

SCHOOL OF PHARMACY

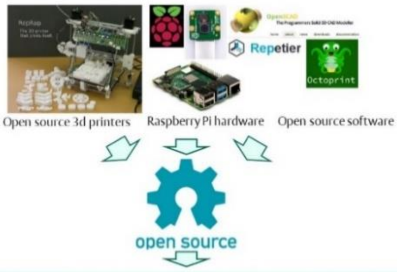
University of Reading

Open Source Hardware: Automated time-lapse for visualising bacterial colony & sugar utilisation with POLIR

Diep The Tai | Sarah Needs | Alexander Daniel Edwards

The Open Hardware revolution: cheap components; free designs and software

Life science is only starting to realise the benefits of a revolution in open technology. Low cost high performance electronics, robotics and optoelectronics components are freely available, resulting from consumer-driven mass-manufacture. For example, Raspberry Pi singleboard computers (<$40) and camera (<$30) and Arduino microcontrollers (<$20) use microchips and CMOS sensors from consumer products (i.e. smartphones) to miniaturise powerful computing tasks. Open source software allows rapid exploitation of this hardware without bespoke programming. Open source 3D printing has proven the power of this approach. A new revolution in **open source lab hardware** exploits such components to deliver powerful lab tools such as 3D printed digital microscopes e.g.: https://openflexure.org/ and https://open-labware.net/



Open source lab hardware
Accessible & affordable lab robotics and automation

Need for high throughput microbiology

Existing lab equipment (e.g. microplate or Biolog reader) is expensive but restricted to specific applications. Lab automation (e.g. robotic workstations) for high-throughput studies can be prohibitively expensive, or too specialised for use in diverse research applications. This has limited the availability of automation that would be very useful for a wide range of life science assays and microbiology research, ranging from antimicrobial screening, through identification, to serology and biology.

Solution: POLIR- <$500 open source high throughput lab automation robot

POLIR (Raspberry Pi camera Open-source Laboratory Imaging Robot) is an open source hardware tool for high throughput kinetic screening of a wide range of life science assays. It can be used in classical microplate and petri-dish solid agar analysis, such as colony growth or sugar utilisation kinetics[1-3]. To date we have used it to explore the effects of exogenous agents on bacterial growth, such as profiling antibiotic resistance and sugar utilisation. It is also compatible with microfluidics- we recently found it invaluable for optimising microdevice assays, and were able to analyse >2000x 1uL microfluidic tests in a single overnight experiment[4] delivering thousands of growth curves.

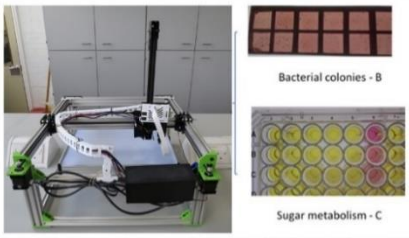


Figure 1: A The POLIR robot has 300x420mm white light bed for analysis of a wide range of analytical microbiology tests including: B colony growth on 3D printed dip-slide frame, C sugar utilisation assay in microplate performed using phenol red pH indicator.

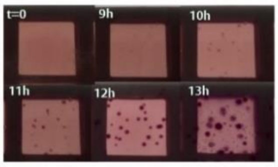


Figure 2: Time lapse of colony appearance on dip-slide. First colonies appear at 9h then grow rapidly over subsequent 4h, showing detail kinetics.

Figure 3: Time-lapse of sugar metabolism from 0-5 hour incubation (1h intervals) with pH changes indicating kinetics of sugar utilisation.

Features and conclusion

- Fluorescence or colorimetric imaging in any format
- Web interface allowing checking experiments real-time online
- Fully programmable x,y,z,t image acquisition
- High-res camera allowing resolution down to micron scale
- Range of magnifications to wide angle, lens configurations
- Rapid, free customisation of open 3D-printed components
- Built-in incubator option allows precise temperature control
- Integrated analysis possible onboard using open software

POLIR is an inexpensive yet powerful tool for standard and novel microbiology research that can deliver large quantities of detailed kinetic data for multiple microbiological assay formats. The open source hardware model allows flexible, inexpensive laboratory instrumentation to be developed that combines the latest high-resolution digital cameras and computing modules with precise x-y-z motion robotics originally developed for 3D printing.

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6.2. Full paper

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The extent of Contribution to research

In this work, I:

1. Performed the microbiology test
2. Wrote the methods for microbiology test
3. Collected and analysed the data for the microbiology section

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RESEARCH ARTICLE

Exploiting open source 3D printer architecture for laboratory robotics to automate high-throughput time-lapse imaging for analytical microbiology

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Abstract

Growth in open-source hardware designs combined with the low-cost of high performance optoelectronic and robotics components has supported a resurgence of in-house custom lab equipment development. We describe a low cost (below $700), open-source, fully customizable high-throughput imaging system for analytical microbiology applications. The system comprises a Raspberry Pi camera mounted on an aluminium extrusion frame with 3D-printed joints controlled by an Arduino microcontroller running open-source Repetier Host Firmware. The camera position is controlled by simple G-code scripts supplied from a Raspberry Pi singleboard computer and allow customized time-lapse imaging of microdevices over a large imaging area. Open-source OctoPrint software allows remote access and control. This simple yet effective design allows high-throughput microbiology testing in multiple formats including formats for bacterial motility, colony growth, microtitre plates and microfluidic devices termed 'lab-on-a-comb' to screen the effects of different culture media components and antibiotics on bacterial growth. The open-source robot design allows customization of the size of the imaging area; the current design has an imaging area of ~420 × 300mm, which allows 29 'lab-on-a-comb' devices to be imaged which is equivalent 3480 individual 1μl samples. The system can also be modified for fluorescence detection using LED and emission filters embedded on the PiCam for more sensitive detection of bacterial growth using fluorescent dyes.

Introduction

Traditional methods of microbiological screening are time consuming, laborious, resulting in high costs for time and labour. Even within clinical microbiology labs, where a large number of samples are processed, automation remains low [1, 2]. The use of high-throughput automated systems allows increased sample processing, cost saving, and more flexibility in testing,

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Competing interests: ADE is one of the inventors of patent application 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 application: WO2016012778 "Capillary assay device with internal hydrophilic coating" AD Edwards, NM Reis. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

for example screening against exogenous agents such as antibiotics for antimicrobial resistance (AMR) [1, 3]. Microbiological experiments deal with a high variety of sample formats such as soil samples and medical samples, including sputum and urine, and the protocols for analyses can be varied: from microtitre plates and agar plates, to microscopy and bacterial identification strips such as API strips. For this reason, platforms for measuring microbiological experiments tend to be highly specialised for a specific experiment, such as Biolog's microbial identification system, or of limited use, as flexibility is difficult to achieve. While automation and partial automation of microbiological techniques may improve sample processing time, the techniques used for many experiments remain unchanged, using classic agar plate growth to culture bacteria and phenotypically study aspects such as antimicrobial resistance. Antimicrobial resistance has been identified as a global threat to human health and existing techniques to identify antimicrobial resistance remain too slow and costly to warrant a test before treatment of a patient begins. This can have a broader negative impact, through the build-up of antimicrobial resistance through over-use of antibiotics and incorrect selection leading to treatment failure and driving maintenance of resistance [4–6].

To combat this issue, improved techniques are required which provide a higher throughput and more flexible scope of analysis than traditional methods. Microfluidic techniques have been used to study bacteria phenotypes, to detect pathogenic species, and measure antimicrobial resistance at speeds approaching those required for a point of care device [7–11]. We have described a simple, low-cost microfluidic device that can be used to measure multiple antimicrobial resistance profiles of bacteria using the metabolic sensitive dye, resazurin [12] that detects bacterial growth by colour change from blue to pink. This test allows high throughput microfluidic devices, termed 'lab-on-a-comb' that are compatible with existing laboratory equipment, 96 well microtitre plates. These devices are made from a melt-extruded highly transparent fluorinated ethylene propylene co-polymer (FEP-Teflon®) microcapillary film (MCF) and comprises a ribbon containing an array of 10 capillaries along its length with an average diameter of 206 ± 12.6 μm. Test strips consist of a 33 mm length of MCF with each capillary internally coated with a different antibiotic of choice to test [13]. Therefore, a single well of a microtitre plate can be expanded to test for up to 10 antibiotics or 10 concentrations of a single antibiotic. While the test relies on a simple colour change and images can be analysed taking colorimetric or fluorescent images, no such reader analogous to a plate reader exists. Furthermore, to aid in the development of new technology to measure bacterial phenotypes, detailed information on bacterial growth, morphology and kinetic effects of substances is beneficial. To collect this data manually is time consuming and labour-intensive (i.e. manually taking images every hour), and therefore systems are needed that increase the automation of the analysis of microfluidic devices, and that increase sample throughput.

The development of new scientific equipment is costly and time consuming, and is repeated by laboratories all over the world, in order to achieve suitable capabilities without the high costs of proprietary scientific equipment. The time spent independently developing and re-developing these techniques hinders scientific progress globally and limits the ability of many facilities to participate in some areas of research [14]. The open-source hardware movement aims to aid rapid scientific progress by increasing the accessibility of laboratory hardware designs globally, and allowing scientists to share, utilise and improve upon hardware designs. This allows flexibility in design for specific technical requirements, giving scientists the ability to tailor their laboratory to their needs, and enables low-cost innovation in scientific methods. Growth in open source hardware designs combined with the low cost of high performance optoelectronic and robotics components has allowed a resurgence in in-house custom lab equipment development [15–19]. The use of open-source hardware in biological imaging has been successfully implemented in the use of microscopes to overcome the alternative costly

and inflexible laboratory equipment, such as the use of a Raspberry Pi computer and camera alongside Arduino-based optical and thermal control circuits for microscopic monitoring of model organisms [20]. The Raspberry Pi camera has likewise been combined with an elegant x-y-z micron precision stage that exploits the flexibility of a 3D printed polylactic acid plastic frame to deliver a versatile, compact, 3D printed open source digital microscope [21].

The concept of an open-source, low cost 3D printer capable of printing the majority of its own components was proposed by Adrian Bowyer [22], and is known as the RepRap movement (replicating rapid prototype). The RepRap movement led to a massive expansion of accessible, low-cost high-performance 3D printers. This open-source hardware has been utilised as an affordable method of 3D printing laboratory equipment by scientists in various fields [23–25]. We have exploited RepRap 3D printer architecture to develop a low cost (below $700), open-source, fully customizable robotic high-throughput imaging system for analytical microbiology applications called POLIR (raspberry Pi camera Open-source Laboratory Imaging Robot). The frame and x-y-z motion is designed around simple v-slot aluminium extrusion widely available across the world and allowing simple customisation. This system is capable of taking time-resolved images of large panels of samples of different microbiological based assays that use petri dishes, microtitre plates and microfluidic devices, with time intervals of as little as seconds, and total experimental times of up to days. Using existing open-source 3D printer designs published under creative commons licences, the frame was adapted to hold a Raspberry Pi camera attached to a z-linked actuator to adjust z-height, replacing the extrusion head. All resources developed are available under open-source licenses and are deposited in GitLab.

While several instruments already exist for use in specific microbiological experiments they are usually designed for a single purpose, i.e. they can only study one petri dish at a time [17] or microtitre (MTP) plates of a certain size but not both [26]. Furthermore, these may only be used for endpoint experiments and not suitable for using in experiments that aim to determine kinetic parameters. The POLIR system is designed to take images over an area of 300 × 420 mm, with larger frames possible by simply using a longer aluminium extrusion frame. Due to the simple design, many different microbiological assays using colorimetric or visible detection can be measured in a single experiment. The simple adaptation by addition of high-power single-wavelength coloured LEDs and emission filters permits fluorescent imaging. The z-axis allows images of samples at different heights to be collected without modifying zoom or camera focus, simplifying experiments where different culture formats are run in parallel (e.g. agar plate plus microfluidic device). This system sits in a walk-in 37˚C incubator room to maintain temperature, however, it is possible to add a heated enclosure around the imaging area if an incubator room is not available.

While other equipment exist for monitoring bacterial growth over time such as heated plate readers that take measurements over time, these systems can only measure one plate at a time, a total of 96 samples. Other analytical microbiology systems, such as Biolog and the Omnilog reader, support bacterial cell phenotyping using preloaded 96 well plates and a reader that allows kinetic analysis of single plates or up to 50 MTPs (4800 samples), monitored every 15 minutes and includes software for data analysis [26]. While these systems already exist and prove the value of kinetic microbial growth analysis, the instrumentation is not widely available to all labs and are restricted to specific proprietary plates and experimental formats. The POLIR platform can monitor 10 plates (960 samples), and a larger frame would easily permit more plates to be monitored for only the cost of longer aluminium extrusion.

A key application for flexible time-resolved automated imaging is for optimising microbial detection and antimicrobial resistance measurement, important for a wide range of applications including healthcare (e.g. infection, pharmaceutical manufacturing) and environmental

(e.g. antimicrobial resistance spread from agricultural use of antibiotics). Development of rapid AMR tests would ideally measure bacterial resistance to antibiotics directly from the sample. In many cases a change in sample matrix can affect both microbial detection and antibiotic activity. For example, for use in the dairy industry it would be ideal to analyse milk samples from dairy cows with mastitis for antibiotic resistance; however, milk powerfully scatters light and is therefore likely to strongly affect colour-based or fluorescent growth detection. Furthermore, milk components may potentially absorb antibiotics (reducing activity) but conversely may in some cases increase microbial sensitivity to antibiotic action, if antimicrobial components are present within milk. It consequently becomes important to study the effect of sample matrix on microbial growth. We therefore used this example to explore whether high throughput analytical microbiology with POLIR can accelerate the analysis of sample matrix interference on antimicrobial susceptibility testing in both conventional and microfluidic test formats.

Materials and methods

Imaging robot design concept and assembly

We assembled a Core X-Y RepRap 3D printed frame with a moving distance of 300 × 300 × 130 mm adapting the D-bot 3D printer design [6]. The system is controlled by the open-source software OctoPi running an OctoPrint server. OctoPrint is commonly used to connect remotely to 3D printers and often utilises a webcam or PiCam to monitor printing progress. The 3D printing extruder was replaced by a Raspberry Pi singleboard computer and PiCam module mounted on a vertical linear actuator (built to an OpenBuilds design [27]) to adjust the z-height, allowing camera focus. The PiCam lens was rotated anticlockwise to focus closer than the supplied infinity focus, allowing a range of fields of view and working distances. We selected a working distance of 80 mm that gave a field of view of 96.5 × 72.5 mm. OctoPrint controls the position of the camera using custom G-code, by supplying G-code from the Raspberry Pi to the Arduino mega board used to control the x-y-z stepper motors. The Arduino board firmware was open-source Repetier software that interprets the G-code driving stepper motors via a RepRap RAMPS 1.4 shield. To take images a Python script configured to acquire the required PiCam image settings (e.g. image exposure, resolution, time) is triggered as an executable shell script via serial command embedded within the G-code and executed by the OctoPrint G-code system command plugin. The images are then stored onboard the Raspberry Pi SD card and can be accessed remotely by file transfer protocol (FTP).

The positional accuracy of the POLIR was tested using G-code to replicate an experiment moving over thirteen overlapping areas of the white LED. An USAF 1951 resolution target was included in one of the areas, and the image sequence repeated every 10 minutes, with each cycle moving over the thirteen areas again over 4–16 hours. The robot was either homed or not between each run of thirteen moves. The depth of focus was determined by sequentially imaging the USAF 1951 resolution target at multiple z heights, either on the surface of the LED light box, or on the surface of an agar petri dish (approximately 4 mm deep agar medium). In all cases, the location of a target line was recorded to determine positional accuracy, and the smallest line pairs that could be resolved clearly was recorded to indicate the image resolution.

The designs, hardware links and software links for the POLIR have been deposited in GitLAB (https://gitlab.com/AlEdwards/polir) [28]. The design for the POLIR was based on open-source designs for 3D printers that can be found at [29] along with the design for the linear actuator from OpenBuilds [27].

Microbiology methods

A soft agar motility assay was performed to determine phenotypes of bacteria. Reference strains of *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 12600 were used (LGC group, Middlesex, UK). The bacterial strains were routinely cultivated on lysogeny broth (LB) agar (Sigma-Aldrich) at 37˚C. Agar at 0.8% in LB broth was autoclaved and, once cooled to 50˚C, supplemented with a final concentration of 0.1 mg/mL Triphenyltetrazolium chloride (TTC, Sigma Aldrich) and 1 mL poured in each well of a 12 well sterile plate. Bacteria was grown overnight in LB broth and normalised to 0.5 McFarland standard and diluted to 1 in 10 000. A 1 μL loop was used to stab the agar to three quarters its depth and the bacteria was incubated overnight at 37˚C and images collected every hour.

To study colony growth on agar plates, bacteria were grown overnight in LB media and normalised to 0.5 McFarland standard. The samples were further diluted to give 10^7, 10^6, 10^5, and 10^4 CFU/mL and 10 μL was spread over LB agar (Sigma Aldrich) plate supplemented with 0.1 mg/mL TTC (Sigma Aldrich) followed by overnight incubation and colony counting to determine CFU/mL.

For microfluidic assays in microcapillary film (MCF), each comb consisted of a custom 3D printed MCF holder containing a row of 33 mm long Lab-on-a-Stick [30] test strips at 9 mm pitch to match microtiter plates (S1 Fig). The fluorinated ethylene propylene 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 206 ± 12.6 μm and external dimensions of 4.5 ± 0.10 mm wide by 0.6 ± 0.05 mm thick. For each batch, 1 m MCF lengths were internally coated by incubation with a 5 mg/mL solution of polyvinyl alcohol (PVOH) in water (MW 146,000–186,000, >99% hydrolysed, Sigma-Aldrich, UK) at room temperature for a minimum of 2h [31]. Coated strips were washed with 5 ml of PBS with 0.5% Tween 20 (Sigma-Aldrich, UK) to remove residual PVOH, and dried with compressed air at 2 bar pressure for 20 minutes. For antimicrobial resistance tests 1 m lengths of MCF were loaded with duplicate capillaries of gentamicin or ampicillin (both at 10 mg/mL dissolved in water). The antibiotic was removed using a vacuum pump leaving behind a thin film of reagent[13].

Bacterial inoculums were prepared according to British Society for Antimicrobial Chemotherapy (BSAC) standard for antimicrobial susceptibility tests[32, 33]. Briefly, an overnight culture was adjusted to a 0.5 McFarland, diluted to the indicated CFU/mL concentration in Mueller-Hinton broth (Sigma Aldrich) supplemented with 0.25 mg/mL resazurin sodium salt powder (Sigma-Aldrich, UK). MCF was cut into 33 mm test strips that were dipped using a custom 3D printed holder into the bacterial inoculum in a microtiter plate (S1 Fig) and incubated overnight at 37˚C. Test strips were imaged by the POLIR in an incubator room at 37˚C, with a white background illumination to record colour change of the resazurin dye indicator; a change from blue to pink indicating resazurin conversion following bacterial growth. To study the effect of milk on bacterial growth, sterile milk (Tesco, UK) was added at the indicated concentration and diluted with Mueller-Hinton Broth such that the Mueller-Hinton was always at 1X. To determine if POLIR could be used to measure growth kinetics in MTP, parallel bacterial growth kinetics through colour change was prepared, and recorded in MTP alongside MCF. For fluorescence measurements, resazurin was used at a final concentration of 60 μg/mL.

Mastitis milk samples were collected from the University of Reading Farm. Serial ten-fold dilutions in peptone water (Sigma) were directly streaked on gram-positive and gram-negative chromagar plates (CHROMagar™ Mastitis). Plates were incubated at 37˚C overnight and bacteria were identified based on the colony colour. Isolates were grown overnight in LB media

and diluted 1:200 followed by 10-fold dilutions and growth was monitored fluorescently by the POLIR for 12 h in the presence and absence of gentamicin and ampicillin (loaded at 5 mg/mL in water).

The ends of the MCF were sealed using a custom 3D printed cap filled with silicone grease to avoid sample evaporation. Analysis of MCF microfluidic devices was performed in MatLab and analysis of microtiter plates used ImageJ.

Results

The POLIR was based on a 3D printer configuration known as CoreXY [34]. This open-source 3-axis robot was developed to maximise positional accuracy whilst minimizing cost for rapid and programmable x-y-z positioning of an extruder for fused filament deposition 3D printing, following the RepRap principles of 3D printed components allowing self-replication. To address the microbiology laboratory's need for cost-effective, open-source, programmable x-y-z positioning of a lab imaging camera, we selected this design as a basis for developing an open-source laboratory imaging robot.

This robot was designed to be able to take detailed time-lapse images and record kinetic data for large number of samples whilst decreasing the amount of hands on time for the experimenter, who would otherwise need to make repeated measurements (e.g. in a plate reader or with camera). To be able to cope with large number of samples a moveable camera is beneficial. Using 18 'lab-on-a-comb' devices and a 96 well microtiter plate, images of the whole experimental area were taken using a Canon EOS 1300D with ES-F f/2.8 Macro Lens costing approximately $700, which is the entire cost for the POLIR system. Using a fixed camera for the entire imaging area of the POLIR does not allow for high resolution images. Using the Canon system, each capillary width of our microfluidic device only accounts for 1.5 pixels (Fig 1A). Using a moveable camera allows a shorter focus with each capillary made up of 6 pixels (Fig 1B). Static cameras would also be a problem for imaging multiple formats of different heights. Microtitre plates need to be imaged from above each well or the sides of the well begin to obscure the sample to be measured (Fig 1A and 1B).

A key aspect of increasing the information obtained per experiment with decreased hands on time for the experimenter relies on automated image analysis. Simple image analysis automation such as MatLab scripts are made easier when each image is in the same place every time-point, especially with microfluidic devices with a small measurement region of interest. To determine the suitability of POLIR for automated microfluidic device image analysis, positional accuracy was measured. The POLIR was run through a full experimental setup for 4–16 h imaging every 10 minutes including a USAF 1915 resolution target. For experiments that were homed in the x, y and z axis once at the beginning of the experiments we found that the average distance in movement frame to frame from the mean for x and y-axis is 20 ± 1.36 and 26 ± 1.76 μm respectively. The majority (98%) of data points fell within 100 μm of the mean (Fig 1C–1E), therefore allowing MCF with a capillary diameter of 200 μm to be imaged successfully and followed by automated image analysis. Experiments that were homed at the beginning of each image (every 10 minutes) had a greater degree of movement (Fig 1F). Homing at the start of each image acquisition resulted in an average movement of 35 and 29 μm from the mean. Finally, to confirm resolution and depth of focus, the USAF 1951 resolution target was used to identify z-height for maximal resolution (Fig 1G). These experiments used a working distance of 80 mm and field of view of 90 mm, which gave over 10 mm depth of focus with maximal resolution of 8 line pairs per mm corresponding to 62 μm line width, 4× smaller than the 200 μm capillaries within MCF. All subsequent experiments presented here used this optical setup, however the PiCam is versatile and simple adjustment of the stock lens allows

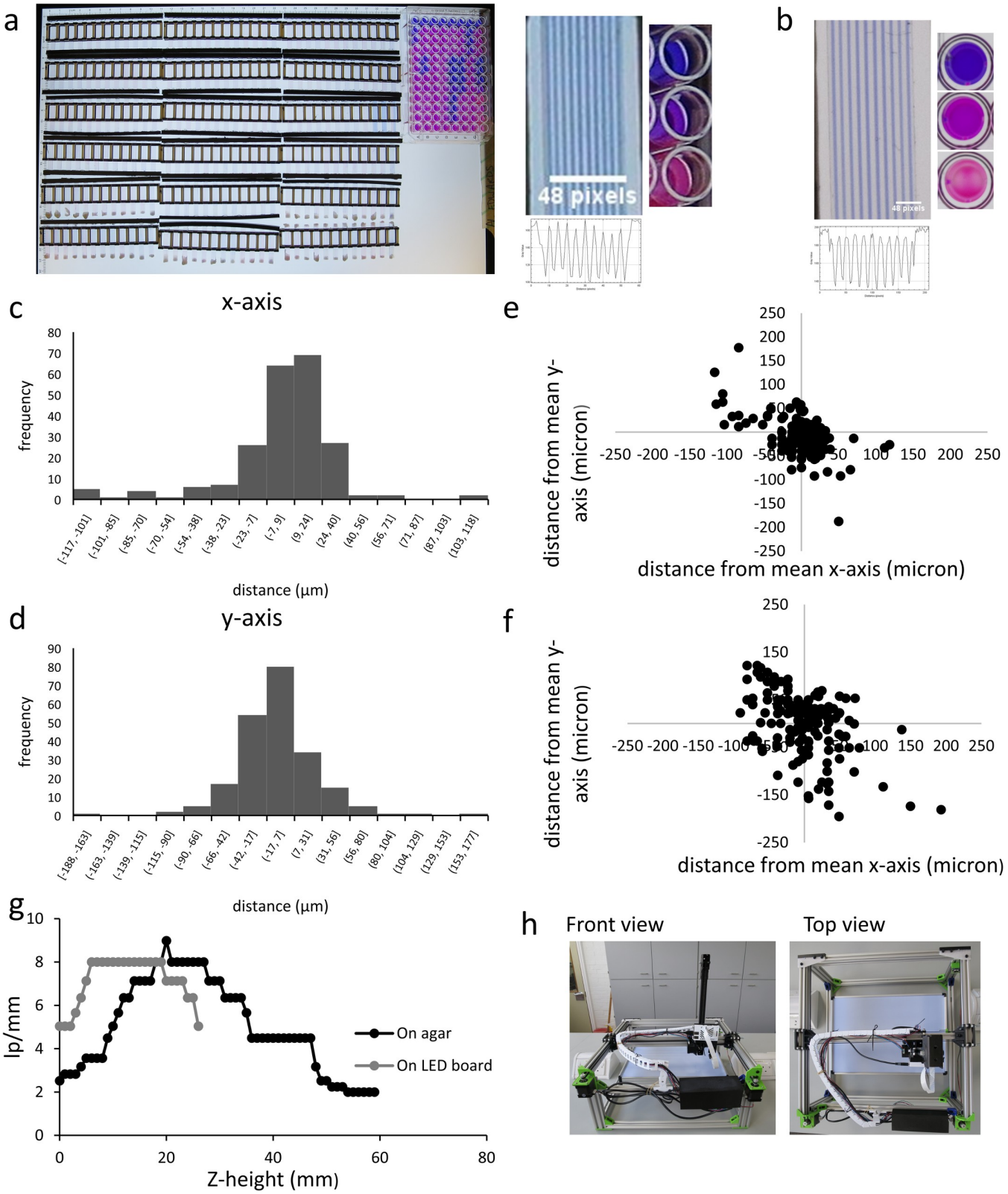

Fig 1. POLIR architecture, positional accuracy, and resolution. A3 imaging size of POLIR filled with 2160 individual capillaries and a 96 well plate. The area was imaged using a Canon EOS 1300D with ES-F f/2.8 Macro Lens (a) or individual images using the POLIR (b). Scale bar indicates 48 pixels. Histogram of the deviation of repeated measurements from the mean for the x-axis (a) and y-axis (b). Scatter diagrams showing the x and y positions in relation to the mean

when there is no homing (c) and with homing every 13 image acquisitions (d). (e) line pairs/mm in relation to the z-height of the camera axis for objects flat on the white light LED board and on agar plates. (f) Image of POLIR from front view and top view. All files needed to build the POLIR have been deposited on GitLAb licenced under the MIT license. (g) Number of line pairs/mm from USAF 1915 resolution target seen at increasing z-height. (h) Photograph showing the front and top view of the POLIR.

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closer working distance with higher magnification and resolution, since the camera is attached to a z-linked actuator it is also possible to image experimental setups with different heights to maintain focus (Fig 1H).

Having established the positional accuracy of POLIR, we wished to test the system for recording microbial movement by imaging motility assays. Motility assays can be used to determine phenotypes of bacteria [35]. Conventional motility assays are interpreted as an end-point after overnight incubation. The POLIR allows detailed measurement of kinetic motility analysis (S1 and S2 Files), with the difference between motile *E. coli* and non-motile *S. aureus* being clear as early as 7.5 h (Fig 2A). Time-lapse imaging increases the quantity and quality of bacterial motility data and would permit detailed analysis of the effect of exogenous stimuli on growth and motility, i.e. delayed or increased speed of motility. This experiment was performed in a single 12-well plate; if the full imaging area of the POLIR was used, this would allow 10 × 12 well plates giving a total of 120 conditions that could be screened simultaneously. The POLIR can monitor colony growth to determine speed of growth and colony morphology. *E. coli* 25922 was grown on LB agar supplemented with the insoluble formazan dye, TTC, used to increase bacterial colony contrast against the white light brightfield format (Fig 2B and S3 File). Simple image manipulation to make the images binary aids in morphology mapping and allows colonies to be detected earlier and much smaller giving more detailed information on size, shape and growth over time.

The rise in antimicrobial resistance has been partly exacerbated by the injudicious of antibiotics. This can be attributed to traditional techniques proving too slow to provide a diagnosis before treatment is prescribed [6].The possibility of a direct POC antimicrobial test is therefore appealing [36–38]. One such test that could be used to improve selection of the right antibiotic and minimise excessive use of antibiotics is against bovine mastitis. Mastitis is the infection of the mammary gland and treatment involves antibiotics. However, due to the spread of antibiotic resistance many infections are not simple to treat [39]. Microfluidic devices are well-placed for use in diagnostic testing due to their small size making them more portable than other techniques. We have explored the use of MCF as a diagnostic for antimicrobial resistance in dairy cow mastitis. One of the major pathogens that causes mastitis in dairy cattle is *E. coli* [40]. To be able to directly test AMR in milk samples the effect of milk as a matrix for bacterial growth was needed. Bacterial growth was measured using the metabolic sensitive dye, resazurin. By using the POLIR we can test side by side the MCF device MTP with kinetic data of *E. coli* 25922 growth in increasing concentrations of milk.

This gives valuable information, if only the beginning and endpoint results were taken it would be unclear whether concentrations of milk higher than 50% have differences dependent on bacterial count concentration as the no bacterial control still converted the resazurin but at a much slower rate (Fig 2C). Milk concentration also affects the absorbance measurements in MCF to a greater degree than in MTP indicating samples would have to be diluted to determine bacterial growth in MCF. This is due to the decreased pathlength in the MCF compared to the MTP which decreases the colour intensity. At the same time, milk contains more light scattering particles making the sample appear opaque [41]. This decreases the range between the starting blue and endpoint pink. There is also a general decrease in absorbance in MCF with no bacteria which is exacerbated by increasing milk concentrations. While a small

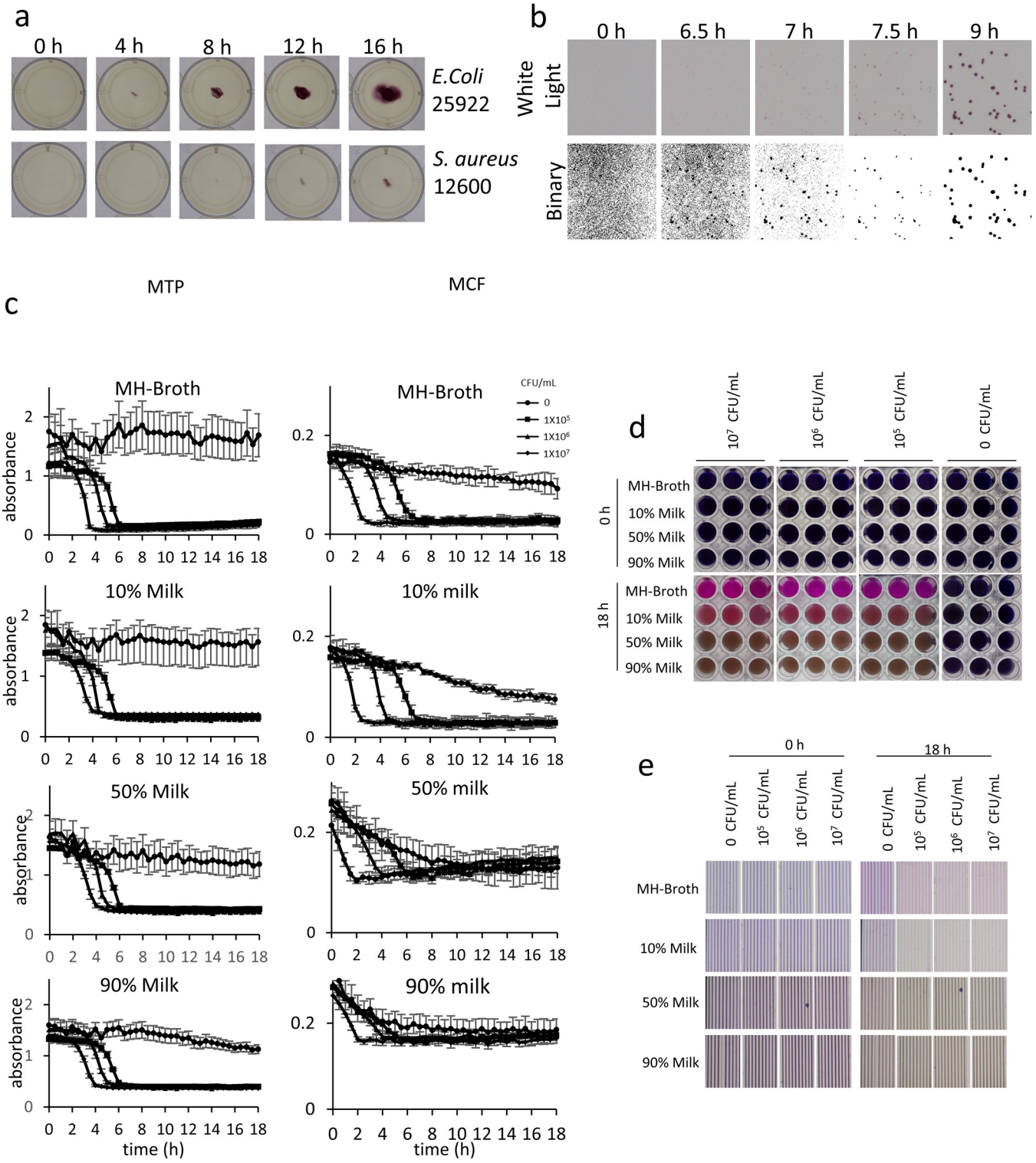

Fig 2. Demonstration of different experimental formats performed using POLIR using agar plates, microtitre plates and microcapillary film. (a) Motility assay using motile *E. coli* 25922 and non-motile *S. aureus* 12600. (b) Detection of *E. coli* colonies on LB agar supplemented with 0.1 mg/mL TTC showing both the raw and binary images. (c) Effect of milk concentration on *E. coli* growth and detection within MCF, error bars indicate ± SD of 3 replicate wells for MTP or 10 capillaries for MCF. (d) Images of MTP wells at start and endpoint of data shown in Fig 2C. (e) Images of MCF at start and endpoint of data shown in Fig 2C.

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amount of resazurin may be converted to resorufin in high milk concentrations, this is not seen in MTP due to the longer pathlength, the overall colour remains blue as it has more intensity than in the MCF.

Previous experiments using MCF microfluidic devices have been restricted to manually taking images, due to its non-standard format, restricting the number of data points and time of monitoring from around 7 data points over 7 h. The POLIR allows for monitoring over much longer time periods and depending on the need, imaging as often as every 10 s in a static position or every 15 minutes using the entire imaging area. This allows increased accuracy when modelling growth kinetics.

Different sample matrixes can affect antibiotic function as well as dye conversion. The effect of increasing concentration of milk on bacterial (*E. coli*) resistance a single concentration of gentamicin milk was tested using the POLIR in microtitre plate. While there was no visible effect on resistance profile in 10% milk when compared to Mueller-Hinton broth, concentrations higher than 50% showed colour change in conditions even in the presence of gentamicin. This was reflected in MCF indicting that any direct testing of AMR in milk would have to be diluted to a minimum of 1 in 10 before testing (Fig 3).

To increase the applications of the POLIR the system can be modified for fluorescence detection using low-cost LEDs and coloured glass emission filters embedded on the PiCam for more sensitive detection of bacterial growth using fluorescent dyes (Fig 4). Metabolism of resazurin changes colour from blur to pink but also from non-fluorescent to highly red fluorescence compound, resorufin. Fluorescent detection of resazurin also allows a smaller amount of dye to be used in the microcapillary film going from 250 μg/mL to 60 μg/mL while still being visible (S2 Fig). Fluorescence detection of resazurin conversion is more sensitive than absorbance measurements and increases speed in detection of *E. coli* 25922 by approximately 2 h. The current design only allows a single fluorescence wavelength to be measured at one time, but the system is flexible enough to change the LEDs and filters to the desired wavelength of choice.

The POLIR was used to screen 24 bovine mastitis samples against gentamicin and ampicillin. Ampicillin and gentamicin were loaded into duplicate capillaries and tested against four dilutions of bacteria and growth was detected by fluorescent intensity of resorufin. Isolates were tested with duplicate strips with four ten-fold dilutions. Three of the isolates were tested using overnight liquid culture or resuspension of colonies from a fresh agar plate. A total of 216 test strips corresponding to 2160 individual capillaries were measured every 30 minutes for 12 h (S3 Fig). Collecting kinetic data of multiple dilutions allowed the doubling times of the isolates to be calculated while simultaneously measuring susceptibility to ampicillin and gentamicin (Table 1). The different isolates had different effects on resazurin metabolism (S3 Fig). Resazurin has been proposed for high-throughput screening for antimicrobial assays due to it's metabolism into the highly fluorescent resorufin [42–44], however, resorufin can be further metabolised into the non-fluorescent dihydroresourfin [42] leading to a transient fluorescent signal in some systems. Transient fluorescent signals can be seen in a number of the mastitis isolates (S3 Fig) and is not-related to generation time. Using end-point measurements would lead to misinterpretation of results.

Discussion

Open-source laboratory hardware has recently drawn a large community of researchers, with new designs for different applications frequently emerging.

A significant advantage of the POLIR over traditional methods of imaging, is the remote access of the system, which can be monitored and controlled via a computer or smartphone

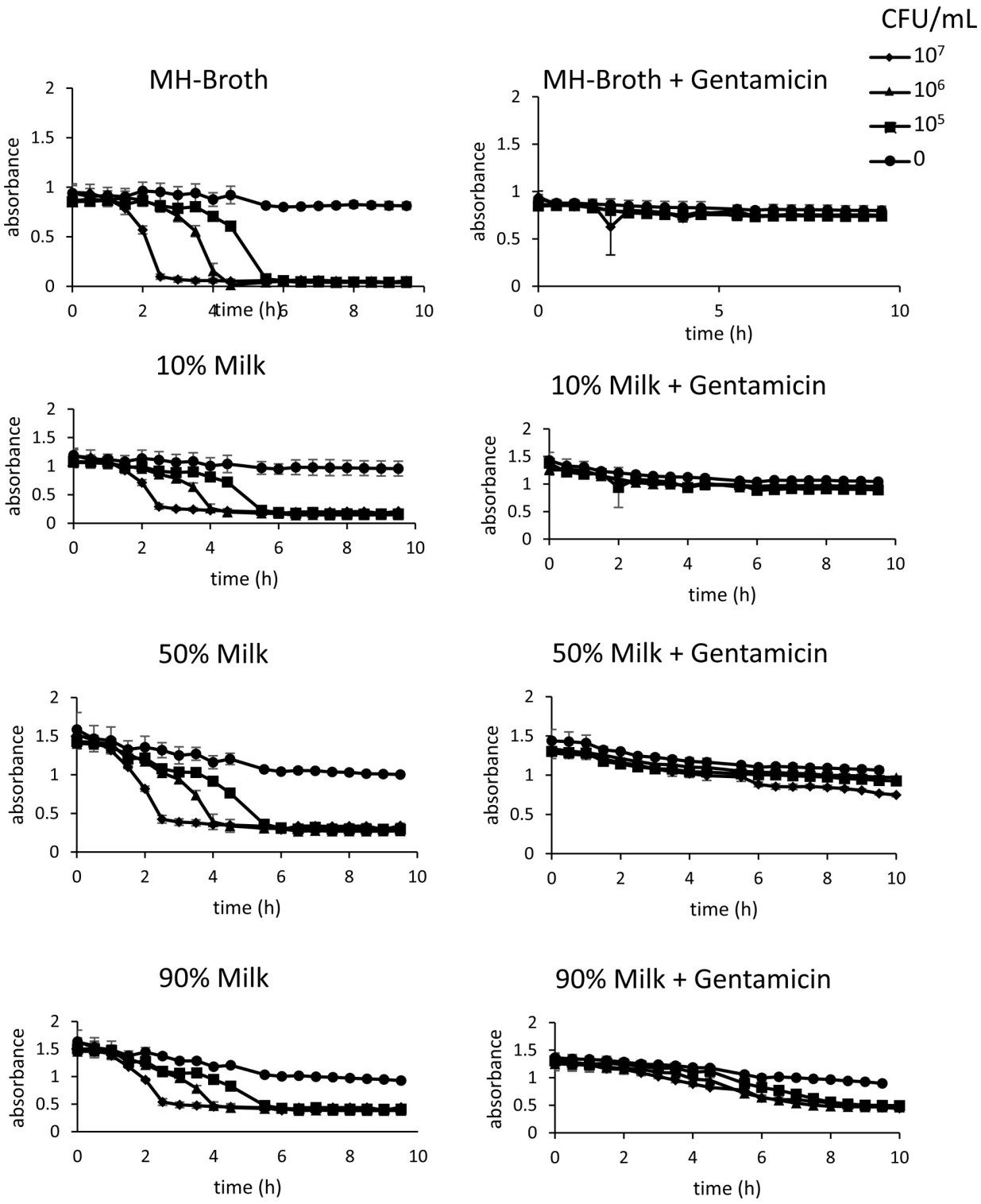

Fig 3. POLIR can be used to screen antibiotic resistance of bacteria. Growth curves of *E.coli* 25922 grown in MH broth or 10, 50 and 90% sterilised milk diluted in MH broth without and with 5 μg/mL gentamicin in MTP. Error bars indicate ± SD of triplicate wells in microtitre plate.

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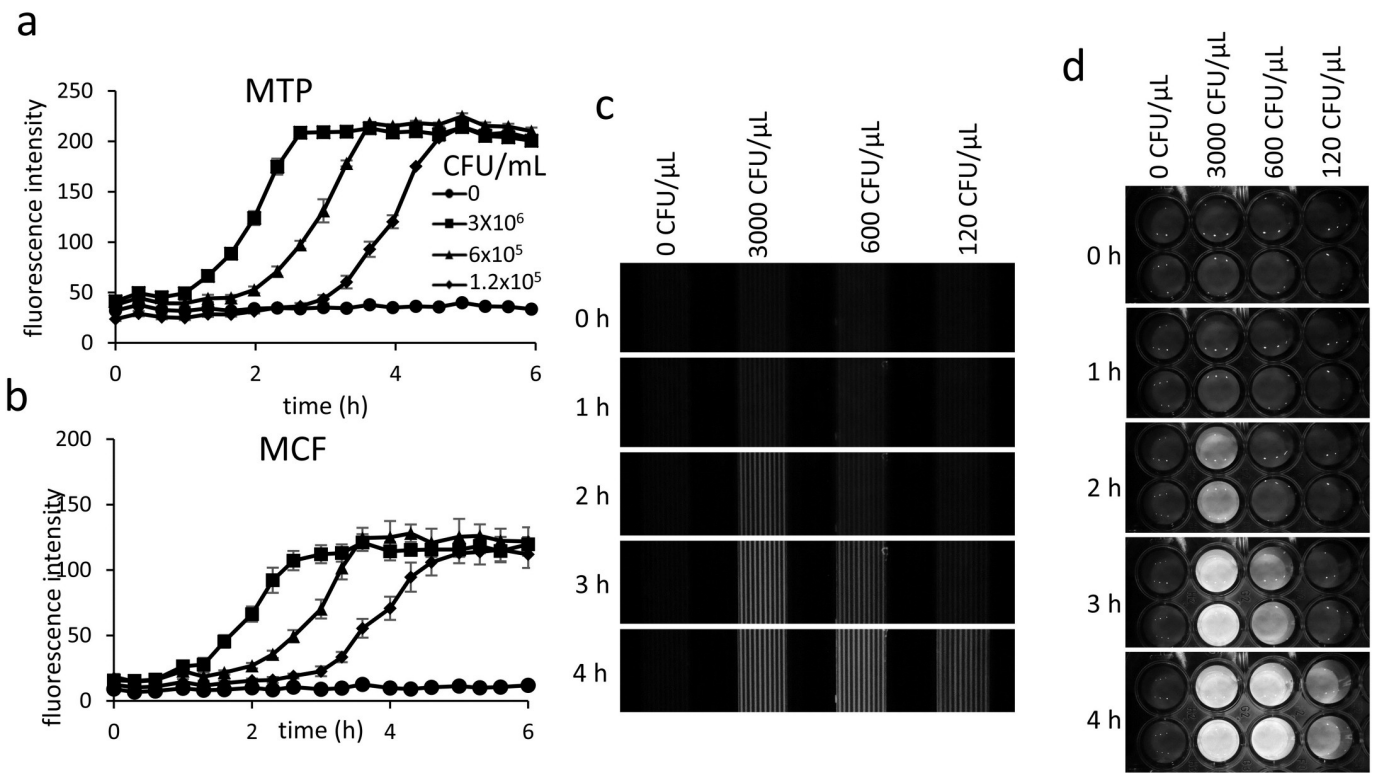

Fig 4. Adaptation of POLIR for fluorescence measurements. Growth curves of *E. coli* 25922 detected by fluorescence increase in resazurin metabolism measured in microtitre plates (a) and micro capillary film (b) Error bars indicate ± SD of duplicate wells in microtitre plates or 10 capillaries in microcapillary film. (c) Images captured from POLIR of microtitre plate and (d) microcapillary film.

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device, giving the researcher flexibility in when they can run experiments and check the progress of an experiment in real time, reducing the need for hands-on time imaging the experiment. Compared to relatively common automated plate readers, the POLIR allows kinetic data collection for multiple plates simultaneously as well as other microbiology-based formats, giving high-throughput, flexible analysis (Table 2). The precise movements of the POLIR results in uniform images over time. Experimental analysis is often one of the bottlenecks in research labs dealing with image analysis. One important aspect of analysis automation is to keep images in the same location every time. When taking images manually using a camera it is nearly impossible to get the frame exactly right. However, using the POLIR most images are within 50 μm of the mean and when looking at peak heights a small variation control is an easy addition in analysis programmes. This is especially important when imaging small devices, such as MCF where each capillary is only 200 μm wide. Automation allows large number of images to be analysed in the time it takes to analyse a single image manually.

This locational accuracy allows for rapid image analysis as positional inputs for colorimetric of fluorescent data do not need to be manually assigned for each image, even laboratories not using programming for image analysis such as MatLab or Python can take advantage of ImageJ region of interest manager which is routinely used in life sciences laboratories.

The main limitations of POLIR are the need for specific laboratory conditions. The current design for the POLIR does not include in a built-in incubator and experiments are performed in a walk-in incubator room which is not available to all laboratories. Incubators can be easily adapted to work alongside POLIR, either within the frame using heating elements and a clear lid, or by placing POLIR inside an incubator.

Table 1. Doubling time and resistance/susceptibility profile of bacteria isolated from bovine mastitis samples.

Isolate Number	Isolate Code	Doubling Time (minutes)	Gentamicin	Ampicillin
1	19.02 MRF GN	23	S	S
2	19.02 MRF GPC blue	33	S	S
3	19.02 MRF GNC white	*nd*	*nd*	*nd*
4	19.05 MRF GPC blue	19	S	S
5	19.16 MRF *S. uberis*	37	S	S
6	19.13 MRF Proteus	31	S	S
7	19.10 MRF PSA	29	S	S
8	19.08 MRF SA	26	S	S
9	19.10 MRF *S. uberis* (13)	29	S	S
10	19.06 MRF GPC blue	26	S	S
11	19.16 MRF EC	15	S	S
12	19.15 MRF SA	26	S	S
13	19.13 MRF SA	*nd*	S	S
14	19.11 MRF	*nd*	*nd*	*nd*
15	19.02 MRF LC+	18	S	S
16	19.02 MRF L-	25	S	S
17	19.12 MRF	*nd*	*nd*	*nd*
18	19.10 MRF S. uberis	33	S	S
19	19.17 MRF EC	13	S	S
20	19.17 MRF	*nd*	*nd*	*nd*
21	19.14 MRF S. uberis	*nd*	*nd*	*nd*
22	19.14 MRF KLEB	49	S	R
23	19.14 MRF SA	*nd*	*nd*	*nd*
24	*E. coli* ATCC 25922	16	S	S

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The open-source robot design allows customization of the size of the imaging area; with the current design, with an imaging area of ~300 × 420 mm, if custom MCF holders with a 6 mm pitch are used a total of 432 MCF strips can be imaged giving a total of 4320 individual 1 μl samples. The current configuration 3280 × 2464 pixel image has a field of view of 96.5 × 72.5 mm with 29 μm/pixel, providing a total imaging resolution exceeding 14,000 × 9800 pixels over an A3 light box. This level of magnification is useful as it provides enough resolution to see individual microcapillaries of MCF with a diameter of 200 μm while allowing at least 4

Table 2. Summary of POLIR benefits over microplate reader or static camera.

Characteristic	POLIR	Microplate reader	Static camera
Throughput	High: number of plates or MCF only limited by size of frame chosen	Low: one plate at a time with no option for other formats	High: number of plates limited by resolution and distance of camera
Image analysis	Can be automated	Highly automated	Can be automated
Resolution	High	NA	Low: obscured wells at edge of image also lowers quality
Flexibility	High: can image anything	Low: only used for microplate assays	High: can image anything
Hands-on time	Low: camera is automated and can be controlled remotely	Medium: each plate must be placed into the reader for each time-point unless the temperature can be controlled	Low: camera can be set up for time-lapse
Data quality	High quality image for each capillary, well or colony	High quality data for each well	Low quality or no data for MCF capillaries and decreasing quality for microplate wells towards the edge of the image

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strips to be in view, decreasing the number of total images need to be taken per experiment. It also allows multiple wells to be viewed when imaging microtitre plates. Higher magnification imaging can be easily achieved by simple modification of the PiCam optics that allows closer focusing. While the size of POLIR can be adjusted for any experimental area, the time taken to image each location will increase with experimental area, and therefore the image frequency interval for each location will increase.

In conclusion, the POLIR is a low-cost imaging tool that allows the recording of colorimetric and fluorescent based microbiology experiments. The POLIR increases throughput and data density of experiments with no increased hands-on time of the experimenter. The simplicity of the design makes the POLIR amenable to adaptation for different experimental needs and the open-source nature allows it to be easily accessed and customized by other researchers.

Supporting information

S1 Fig. Microcapillary film experimental setup. Custom 3D printed holders with 9 mm pitch hold the 33 mm microcapillary film (a). The holders are compatible with 96 well microtitre plates allowing each well to be expanded into 10 capillary tests (b). The ends of the microcapillary film are sealed with a 3D printed cap filled with silicone grease to stop evaporation (c). Multiple test strips in holders are placed on the POLIR to measure either absorbance or fluorescence (d).
(TIF)

S2 Fig. Effect of resazurin dye concentration on speed of detection. *E.coli* 25922 was grown in Mueller-Hinton broth supplemented with the indicated concentration of resazurin. Fluorescence intensity is normalised to the starting intensity. Mean indicates average of 10 microcapillaries. Error bars indicate ± SEM.
(TIF)

S3 Fig. Fluorescence conversion of resazurin by isolates form dairy cattle mastitis samples with and without gentamicin and ampicillin. Negative indicates no antibiotic, ampicillin and gentamicin (loaded at 10 mg/mL). Isolates were grown overnight and diluted 1:200 diluted in Mueller-Hinton Broth with 0.06 mg/mL resazurin followed by 10-fold dilutions. Images were taken every 30 minutes. Negative indicates average of 6 capillaries, ampicillin and gentamicin are the average of 2 capillaries.
(TIF)

S1 File. Timelapse video of *E. coli* motility test presented in Fig 2A.
(ZIP)

S2 File. Timelapse video of *S. aureus* motility test presented in Fig 2A.
(ZIP)

S3 File. Timelapse video of data presented in Fig 2B.
(ZIP)

S4 File. Timelapse video of data presented in Fig 4B.
(ZIP)

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CHAPTER 7 - DISCUSSIONS OF THE WHOLE THESIS

Many innovation technologies approaches have been created during the last decade with a major impact on public health and patient care as well as to restructure and change the role of the microbiology laboratories which is the first line to discover antimicrobial resistance, outbreak response of infectious diseases, and assurance of biosafety, potential bioterrorism (104, 105). Despite these crucial roles, the utilization of laboratory testing results is at least as important for clinical physicians (106-109). Meanwhile, it is unavoidable to laboratory test data influences 70% of medical decisions (110, 111) and improved informative decision-making in healthcare (112). In, this turn, early diagnosis contributed to reducing the lengths of hospital stay, mortality, and admission cost (108, 113). For instance, late identification of blood pathogens and delay of treatment with antibiotics increased mortality 8% with every hour (114). A recent study published by Ngo and colleagues they emphasized that the majority of inpatients (98%) were subjected to at least one or more laboratory tests, and the percentage of test orders decreased in the emergency unit (56%) and outpatients (29%) (110). In contrast, some research showed that unnecessary tests for patients accounted for 40 - 60% (115, 116) and overutilization of diagnostic testing contributed to healthcare expenses and seems to be harmful to patients (117, 118). This controversy has been increasing pressure on microbiology laboratories that face technological innovation for diagnostics, funding investment for doing research or restructuring workflow, space and working environment, staff training, infrastructure, facilities, and strength of the laboratory capacities through quality management systems. There are always, however, gaps between laboratories' sources and requirements of public health. To cope with these matters, some laboratories choose to use centralized laboratory services to solve the problem of standardizing the laboratory data (105), or Mini - Lab with its quality standard worked to another laboratory, focused on robustness, ease of use, easy installation (119), and quality-assured which is a fundamental tool to increase the reliability of test results and improve

patient safety (118, 120, 121). Currently, the trends of transforming conventional laboratory platforms - manual into semi or automatic platforms - have been revolutionizing healthcare (122-124). Although there is no doubt of the benefit and breakthrough of automation, however many tests required highly trained, skilled staff to interpret the results (125), they are time-consuming, and required manufacturers – introducing conflict in supplying software and reagents (123, 124) as well as one - size - fit - all paradigm that does not apply for all laboratories, especially for small laboratories in term of installing space and remote areas (123). In addition, the connection of multiple diagnostics into one single track has been proven that efficiency and standardization have been alternative approaches to the strengthening testing capacity of the laboratory (123). Therefore, the availability of ready-to-use kits that are mobile, do not require much skilled training, can be produced at a competitive price and are space-saving, are priorities for selection by microbiology laboratories. Remarkably, in the emergency of AMR, the need of combining traditional methods and innovative technologies will have to explore novel paths to monitor the AMR and use its data which is another challenge for the microbiology laboratory in the data control and information management to design action plans to control AMR (126, 127). At present, detecting phenotypic antibiotic resistance has remained the gold standard to measure the impact of AMR spreading (127, 128).

Fast, reliable, low cost and real-time diagnostic have still the main scope of development of a new assay for rapid detection of AMR. According to CLSI and EUCAST guidelines, phenotypic testing is required for reliable antibiotic resistance diagnostic (37), and solidly based on the report of resistances rates in epidemiological surveillance (129), even the breakpoints of phenotypic resistance depend on the geographic settings (130). Parallel phenotypic, genotypic assay - molecular test such as polymerase chain reaction (PCR), and next-generation sequencing has been performed to detect AMR and is now considered the gold standard (129). Some resistances, however, haven’t had the genetic alteration because of epidemiological conditions which affected bacterial survival and

bacterial stressors. These will impact the way how bacteria respond to antibiotics (130-132). The discrepancy between genotypic and phenotypic has been remarked on *Pseudomonas aeruginosa* isolated from bloodstream infections, with 45% showing phenotypic resistance to the imipenem and meropenem, just some resistant genes carried strains (133). The same phenomenon was also reported on *Staphylococcus aureus* isolated from mastitis that showed the different resistant gene patterns from phenotypic assay to cephalosporin groups (134). The differentiation was also elucidated on *Salmonella* (135), *Klebsiella pneumoniae*, a Uropathogenic (136), *Escherichia coli,* and *Salmonella* from dairy cattle faeces (137). Meanwhile, these disagreements have not just been found on strains isolated from humans, and cattle, a recent study on environmental *Vibrio cholerae* Non O1, Non-O139 indicated genotypic antimicrobial resistance needs to be better understood as well (138). The advantage of molecular diagnostics using whole-genome sequencing has broadly revolutionized microbiological applications including tackling AMR (139, 140). The high cost, however, and different assembly approaches when performing the genome sequence are other challenges that are to be addressed upon their application for AMR surveillance (141, 142). Despite these challenges, some countries have continued developing strategies actions, and roadmaps for AMR control which are based on this promising tool (141) even still, there is discordance between whole-genome sequence and phenotypic assay (143) and a lack of information to predict antibiotic-resistant evolution (144). Although developing new phenotypic-based assays for early detection of AMR still has some challenges and barriers that need to be addressed, phenotypic-based novel technologies remain the priority for the microbiology laboratory. Since 2015, during the last decade, the development of novel technologies based on phenotype has still dominated, compared to genotypic-based methods in the field, even the application in clinical settings, point of care was still low implemented (figure 7.1). Despite being time-consuming, phenotypic-based methods such as disk diffusion tests or MIC detection still has some advantages such as predicting drug resistance and susceptibility and enumerating the level of susceptibility of

pathogens (145). Whilst the revolution of molecular approaches are exploited in many applications which can't be performed in low-income settings, phenotypic-based assays play a crucial role in antibiotic-resistant surveillance. Recent research showed that using single species to perform susceptibility testing is inadequate to present the resistance situation because the way species interact in the bacterial community is complex and remains unclear. Therefore, the susceptibility test should be performed within complex bacterial communities instead of single species as they are currently (146).

Therefore, we developed a new portable assay based on the type of phenotypic detection which is the current trend globally.

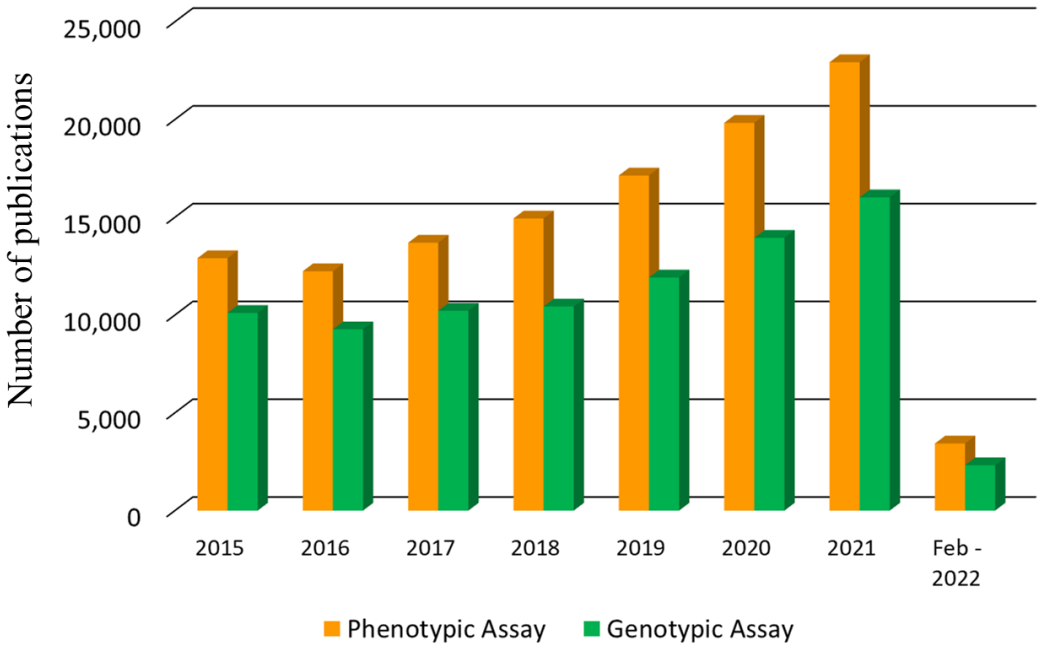

Figure 7.1: Comparison of the publication number in phenotypic vs genotypic antimicrobial-resistant testing. Data generated in full text by search on Google Scholar with search terms "phenotypic based methods for diagnostic bacterial antibiotic resistance" and "phenotypic antimicrobial resistance testing" vs genotypic based methods for diagnostic bacterial antibiotic resistance" and "genotypic antimicrobial resistance testing". Data accessed until February 2022.

To combat the spread of AMR, beyond the inherited innovation technologies, a lack of consumables, reagents, and equipment has led to ineffectiveness in the

control of AMR. Normally, the supply chains of labware for microbiology can take 4 or more weeks to deploy the experiment (78). During the last COVID outbreak, routine laboratory activities have been hugely impacted by supply shortages. Global shipping delays, lack of pipettes, reagents, personal protective equipment (PPE), plasticware, and consumables…have decreased the testing capacity of laboratories (147, 148). The use of 3D printing technology was not only to address these shortages issues, but to offer benefits such as creating the labware without depending on supply chains, or commercial distributors, designing and customizing individual needs, and rapidly developing new methods (78, 148). With recent developments in FFF technology and inexpensive desktop 3D printers, it has become possible for researchers to design what they want and test it in a few hours. This will save time, ease research budgets, and ultimately reduce turnaround time to improve the quality of microbiology. Although we provide examples for plating and replication of bacterial samples to determine MIC on agar, plus dip-slides and plates for multichannel agar media, many different labware components can be readily designed and tested. Other equipment in the microbiology laboratory has also been designed and 3D printed including digital microscopes (149), centrifuges (81), and micropipettes (150). The open publication of these designs support a new wave of microbiology innovation across the globe. With this technology, we designed a new frame dip slide used for the detection of MIC and 3D printed microscopy which is used in the early detection of AMR based on their motility. This tool supports the early detection of antibiotic resistance in the field or beside a patient's bed, contributing to a reduction in the turnaround time and moving the microbiology testing performed in the laboratory into the field – known as deployable diagnostic. Mobile testing trends and portable platform testing proved valuable during the Ebola outbreak, requiring only 4 hours to diagnostic time instead of several days (151). This was no only employed in response to the outbreak, but mobile laboratories were also used for vaccine evaluation and response to major disasters (152, 153).

Furthermore, our 3D printed dip slide can detect both aerobic and anaerobic bacteria directly from samples without any sample preparation needed. With this phenotypic approach, we can directly detect bacterial populations in natural niches and determine their MIC profiles simultaneously, just taking between 9 to 15 hours to get the result. These findings showed that the 3D printed dip slide was able to use both anaerobic and aerobic bacteria. It's easy to perform the test anywhere, with uncomplicated training and introduction for the user. Combining this with our mobile incubator, these portable tests improve the clinical outcome, reduce the significant turnaround time, and can contribute an alternative and rapid way to identify the microbiome with their associated antibiotic resistance profiles.

Incubation at a controlled temperature is a key step in culture-based microbiological tests. Access to culture-based microbiological testing requires access to conventional incubators in a laboratory. Portable incubators allow microbiological testing in the field and resource-limited settings and can eliminate the challenge of sample transportation, minimizing the chance of sample degradation. Here we report that small inexpensive uninterruptable power supply (UPS) products manufactured from consumer electronics and powered by lithium-ion battery packs allowing thermostatic temperature control in small portable incubators that can maintain precise temperatures with or without external power. We present an open-source design for a Microbiological Mobile Incubator (MicroMI) in two sizes for field use. The MicroMI is built from simple and widely available components and is easy to set up. The open-source design can be customized for different numbers of samples. The smallest and most efficient design uses a vacuum insulated food flask that allows longer operation with smaller, lower-capacity UPS. The larger flight case design has space for more samples but depletes the battery faster. The UPS maintains a typical microbiology incubation temperature for up to 24 h without external power- ideal for typical incubation needed for culture methods. The battery capacity, incubator design, and external ambient temperature all affected the duration of operation without requiring external power. We validated the MicroMI by conducting classical

microbiological tests using agar Petri dishes, slant cultures and dip slides, and biochemical tests. The MicroMI design allows inexpensive lithium battery products to be used to simplify field microbiology and increase access to vital analytical microbiology testing.

Determination of bacterial motility is one of the important steps in the identification of microorganisms in microbiological laboratories. Based on the current design of 3D printed microscopy, combined with Raspberry Pi camera model B and used coated with or without polyvinyl alcohol (PVOH) Micro capillary Film (MCF) as well as filling in the semi-solid agar (0.4%) as a carrier for observation of bacteria which compared to slide, hemocytometer on different dye such as bromocresol purple, methylene blue, and phenol red. We developed potential methods for direct detection of vital contaminated bacteria in bottled water, vegetables, and human urine at 10^1 CFU/ml, and 10^5 CFU/ml. As well, with this system, antibiotic susceptibility could be found around 5 to 10 minutes based on non-motile bacteria in liquid media. These initial tests indicate that 3D printed Raspberry Pi digital microscopy combined with microfluidic sample preparation has great potential for direct microbiological analysis of food, water, and clinical samples. The low cost and portability of all components (total materials cost of ~$60, and <200x200mm footprint excluding computer keyboard/mouse/monitor) make this technology accessible for teaching, research, field testing such as investigating outbreaks, as well as diagnostic microbiology in hospital and community settings. Although this is a fast and accurate test, it still has its limitations. This test has only been applied to motile bacteria, non-motile bacteria haven't been tested on this system. There are, however, other platforms using MCF for non-motile that have been developed. Coating the inside of MCF with top agar containing antibiotics, combined with ELISA strip wells, and the use of PiRamid– an imaging device capable of time-lapse imaging, we can detect susceptibility of non-motile bacteria within hours (data unpublished). Meanwhile, using MCF has not only detected the susceptible of the strains, but our system can distinguish gram-negative or gram-positive within the same test. This means using

the MCF system combined with 3D printed microscopy and/or a 3D printed PiRamid imaging rig, allows us to classify gram-negative or gram-positive bacteria and their susceptibility profiles in one test – dip and test.

Growth in open-source hardware designs combined with the low cost of high-performance optoelectronic and robotics components has supported a resurgence of in-house custom lab equipment development. The system comprises of a Raspberry Pi camera mounted on an aluminium extrusion frame with 3D-printed joints controlled by an Arduino microcontroller running open-source Repetier Host Firmware. The camera position is controlled by simple G-code scripts supplied from a Raspberry Pi single-board computer and allows customized time-lapse imaging of microdevices over a large imaging area. Open-source OctoPrint software allows remote access and control. This simple yet effective design allows high-throughput microbiology testing in multiple formats including formats for bacterial motility, colony growth, microtitre plates and microfluidic devices termed ‘lab-on-a-comb’ to screen the effects of different culture media components and antibiotics on bacterial growth. The open-source robot design allows customization of the size of the imaging area; the current design has an imaging area of ~420 × 300mm, which allows 29 ‘lab-on-a-comb’ devices to be imaged which is equivalent to 3480 individual 1µl samples. The system can also be modified for fluorescence detection using LED and emission filters embedded on the PiCam for more sensitive detection of bacterial growth using fluorescent dyes (154).

In low-income settings, these new phenotypic assays have brought a new chance for performing research, especially on AMR detection. Due to low investment and the problem of the supply chain, with issues affecting maintenance and repair services for laboratory equipment, there is a need for robust, low-cost, low and saving energy such as electricity-free incubators (155), and autoclaves powered by solar energy (156). Therefore, using equipment and test kit with low cost, mobile, and saving space for installation is a potential study and runs the microbiological laboratory which contributed to strengthening the surveillance networks of laboratories to improve public health.

In brief, phenotypic-based methods for the detection of AMR are still a priority in the microbiology laboratory. Based on these, we designed a system containing a portable frame dip slide combined with a mobile incubator which supported epidemiological surveillance or performed the microbiological test in the fields to change the turnaround time, to give a chance to use the right treatment for patients. Combining two separate techniques - culture and MIC into one test which is the testing trends and shows promising results. Whether in low-income or developing countries, these kits have contributed an alternative way to control the spreading of AMR. In addition, motility-based methods are also supported for early detection of AMR, just 5 - 10 minutes. This test is also deployed anywhere and is not highly specialised. Automatic image analysis is another solution to give a tool for studying microbiology. By combing 3D printed, Raspberry Pi, we had created another perspective for performing or studying microbiology in new challenging settings with long outbreaks, borderless and effects globally. Our kits also fit the criteria for the ideal test of WHO set up in 2003, which is ASSURED (affordable, sensitive, specific, user-friendly, rapid, equipment-free, delivered) (157).

CHAPTER 8 - CONCLUSION

Our mobile incubator is a piece of portable equipment easily carried to perform tests in various settings including remote areas, and local unspecialised laboratories with limitations of microbiological equipment. Our design has allowed for:

- Use of an uninterruptable power supply (UPS) instead of mains electricity as a power supply.

- Portability to anywhere and combining two or three mobile incubators to increase the capacity of the testing volume.

- Control and flexibility of temperature; easy to set up various temperature points and the heat inside the chamber was stable.

- Space-saving for easy instalment and low-budget investment.

- Applying a wide range of labware such as Petri dishes, tubes, frame dip slides, and plates for any microbiological experiments.

Ideally, we would develop further microbiology labware using 3D printing as well as produce 3D frame dip slides that are affordable for use with this mobile incubator. These two devices produce one kit combining two requirements of microbiological testing for the identification and determination of MIC. It was flexible to self-supply the labware using 3D printed technologies. Some prototypes such as loop, frame and pin replication pin, and squared dish have been produced to demonstrate their application. This labware can also be used the same as routine consumables in the microbiology laboratory.

More interestingly, frame - dip slide, produced from this technology can be combined with two single microbiology tests including the identification and determination of MIC breakpoints in one test. With 3D printed frame, the users in a microbiology laboratory can:

- Reduce the turnaround time to detection of MIC breakpoints from 3-5 days to hours as well as 1 day.

- Directly detect the pathogens and their MIC on samples.

- Be customisable with the frame produced at any desired size.
- Applied with any agar media and panel of antibiotics.
- Combine with mobile incubator into early detection kits for the field
- Reduce the time for preparation of samples and performance of the test.

In addition, using 3D printed technology combined with a Raspberry Pi, we have created new portable kits for early detection of AMR, just 5 - 10 minutes to 10 hours. Although there was a limitation on the type of bacteria, with the test only applying for motile bacteria, this method combined with MCF allowed for:

- Directly detecting bacterial resistance just in minutes
- Use of MCF instead of a glass slide, increasing safety.
- Ease of handling and save cost for investment.

All of these assays are based on a physiological test of bacteria - motility and their response to antibiotics. Battery running incubators instead of mains powered contributed other benefits for saving maintenance and repair to the equipment, but also providing a system that was robust, mobile and accurate, space-saving, low cost that fits the need of most small microbiology laboratories, and also accessible to remote laboratories that wish to perform similar microbiology tests. Ease of use, availability, reliability and competitive pricing are our goals for contributing alternative assays in controlling the problem of antimicrobial resistance, which remains threatening the public health in the world. With this all-in-one test kit, it is expected to deploy in some local laboratories to show more evidence about their advantages is the next step in the future.

In the near future, our proposed future work and next steps will be:

To widely perform 3D printed frame dip slides in the fields where there are limitations in resources.

In Vietnam, local laboratories of hospitals or provinces lacked equipment and investment for microbiology tests. So, most samples must transport to a central laboratory. This affected the treatment time of patients. This 3D frame can

contribute alternative solutions to reducing turnaround time and a customisable panel for screening antibiotics fits the real situation of using antibiotics in the fields.

To perform microbiology tests using 3D microscopy, 3D frames dip slide in a local microbiology laboratory.

The 3D printed frame dip slide is easy to perform, possible to customize and available for long storage. At a local laboratory with the limitation of the number of samples every day, this frame is a good choice because there is no need to buy a full pack reagent with a short expired date. It will be costed and waste. In addition, a combined frame with a mobile incubator is a good solution for the small laboratory where limited spaces and investment in equipment.

To transfer the technology to produce more mobile incubators, not only in medical microbiology laboratories but for application in veterinary laboratories.

The mobile incubator is not only an alternative solution for medical laboratories but also in the animal sectors where there is a need to perform microbiology tests on farms, where on a family farm this could become an essential tool for early detection of pathogens as well. This will help to prevent the spread of disease for livestock, and to save the cost of production if pathogens can be early detected at the site.

Extend measurement of AMR using digital tools to detect motility

Digital tools contribute to the time-lapse detection of pathogens in hours. Therefore, using the camera to capture the image of bacterial growth or motility under the pressure of antibiotics is another assay. This method doesn't cost so much investment and is easy to install and perform.

Combining an incubator with a camera such as the PiRamid system - a hybrid between a mobile incubator and digital camera or light scatter systems - a digital camera system with white light will be parallel methods with 3D microscopy to observe the bacterial antibiotic resistance in hours**.**

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