Antimicrobial Susceptibility Characterisation of *Legionella* in Patients and the Environment

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A thesis submitted in the fulfilment of the requirements of the degree of

Doctor of Philosophy

Cardiff University

School of Medicine, Department Infection and Immunity

2022

ex nihilo nihil fit

Nothing can be made from nothing

Titus Lucretius Carus 99-55BC

Acknowledgements

Firstly, I'd like to thank my supervisor Brad Spiller for supporting both me and my research going above and beyond what I have seen elsewhere in academia, a true role model.

Vicki Chalker, proof that not all civil servants are automatons. Thank you for your constant help. A special mention is necessary here for the Red Pen of Doom, which vastly improved this and all my other work, even if it came at the cost of countless gnomes, trolls, pixies and dinosaurs.

Ian and Kirsty, their friendship, passion for science, passion for beer and passion for potatoes, has been of immense help throughout. This work would not have been possible without either of them.

The YYMCA group of fellow PhD students, Pete, Jack, Roanne, Beth and Owen have collectively made the last four years more than I thought it would be, from bar crawls to lockdown Christmas meals. These friends deserve my thanks for preserving what little remains of my sanity.

The students that I have managed Rupert Kelly, Artjoms Portnojs and George Steadman-Gay also deserve thanks, teaching them, engaging with their ideas and supporting their lab work remains one of the highlights of this project.

James, my best friend, from constantly encouraging me to pursue a PhD, leading by example, to fielding random sequencing-based questions at all hours. Thank you for your unwavering support and belief in me.

Dad, Mum and Mandy thank you, not just for kindling my passion for science but also for unerringly supporting me in my academic career. Nicholas, Emma and Katie, you have always pushed me and the enthusiasm that you have for your respective callings drives and encourages me to do better on a daily basis.

And lastly Barbra, without whom I know without any shadow of a doubt I would not be doing what I am now. She has supported me constantly, from day one to day 1,553, from reading through my work (adding commas left, right, and centre) to helping me streak out isolates (doing so on our anniversary I think qualifies her for beatification in its own right). She brings light to my life.

PHE (UKHSA) deserves significant recognition and thanks for funding my PhD studentship grant, I would like to especially thank them for the additional six months covid relief funding. The T. Maelgwyn Davies Bequest Fund allowed for the additional sequencing of Welsh *Legionella* isolates. As well as an ECDC-funded international collaboration grant: Expert Exchange Programme for public health experts in food and waterborne diseases, Legionnaires' disease and variant Creutzfeldt-Jakob disease (FWDEEP), which facilitated a two-week collaborative trip to Lyon to work in Dr Sophie Jarraud reference laboratory which greatly facilitated this presented work.

Publications arising from this project:

Portal, E. AR *et al*. 2021. *Legionella* antibiotic susceptibility testing: is it time for international standardization and evidence-based guidance? The Journal of antimicrobial chemotherapy 76(5), pp. 1113–1116.

Portal, E. AR *et al*. 2021. *Legionella* antimicrobial sensitivity testing: Comparison of microbroth dilution with BCYE-α and LASARUS solid media. Journal of Antimicrobial Chemotherapy 76(5), pp. 1197–1204.

Patent name and number:

Charcoal-free culture media for *legionella*: WO2020039213 A1

Oral Presentations:

Edward AR Portal, Artjoms Portnojs, Victoria J Chalker, Owen B Spiller: Concordance for MIC results between LASARUS against gold standard microbroth dilution method, while BCYE-α gives elevated and widely variable results. Presented at ESGLI 2019, Athens Greece

Edward AR Portal, Victoria J Chalker, Owen B Spiller: New high-throughput agar-based *Legionella pneumophila* antibiotic sensitivity testing method for large scale. Presented at ESGLI 2018, Lyon France

Poster Presentation:

Edward AR Portal, Victoria J Chalker, Owen B Spiller: Novel high-throughput agar-based *Legionella pneumophila* antibiotic sensitivity a large-scale screening. Presented at SFAM 2019, Manchester UK

Word Count: 34,795

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Table 1 List of Abbreviations

Abbreviation	Meaning
ACES buffer	N-(2-Acetamido)-2-aminoethanesulfonic acid
AMR	Antimicrobial Resistance
ARRCA	Advanced Research Computing at Cardiff
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection (USA)
ATM	Amplification Tagmentation Mix
BAL	Bronchoalveolar Lavage
BCYE	Buffered Yeast Charcoal Extract
BMD	Broth Micro-Dilution
BNF	British National Formulary
BSA	Bovine Serum Albumin
BSYE	Buffered Starch Yeast Extract
BYE	Buffered Yeast Extract
CAP	Community Acquired Pneumonia
CBA	chocolate blood agar
CDC	Centres For Disease Control And Prevention (USA)
CFU	Colony Forming Units
CLED	Cystine lactose electrolyte deficient
CLSI	Clinical And Laboratory Standards Institute (USA)
CSI	Conserved Signature Indels
DAL	Diluted Amplicon Library
DC	District Capital (USA)
ddH2O	Double Deionised Water
DSMZ	Deutsche Sammlung Von Mikroorganismen Und Zellkulturen (German Type Strain Collection)
ECDC	European Centre For Disease Control
ECOFF	Epidemiological Cut-Off
EEA	European Economic Area
Compound E	Error 404
EIA	Enzyme Immunoassay
E-test	Epsilometer Test
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FB (Minion)	Flush Buffer (Minion)
FDA	Food and Drug Administration (USA)
FLT (Minion)	Flush Tether (Minion)
HSE	Health And Safety Executive (Government Body, UK)
HSW	Health And Safety Act Of 1974 (UK)
ICT	Immunochromatographic
ID	Identification
ISO	International Organization for Standardization
IV	Intravenous
IVD	*In Vitro* Diagnostics

LASARUS	Legionella Antibiotic Resistance and Universal Screening
LB	Lysogeny Broth
LB (minion)	Loading Beads (Minion)
LD	Legionnaires' Disease
LNA1	Library Normalisation buffer A1
LNB1	Library Normalisation buffer B1
LNP	Library Normalisation Plate
LNS1	Library Normalisation standard 1
LNW1	Library Normalisation wash 1
Lp Sg 1	*Legionella pneumophila* Serogroup 1
LTM	*Legionella* Transparent Medium
MALDI-ToF MS	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MDR	Multi Drug Resistant
MGW	Molecular-Grade Water
MH	Muller Hinton
MIC	Minimum Inhibitory Concentration
MIC_{50}	Minimum Inhibitory Concentration of 50% of test isolates
MIC_{90}	Minimum Inhibitory Concentration of 90% of test isolates
MLST	Multi Locus Sequence Type
MOPSO	2-Hydroxy-3-morpholinopropanesulfonic acid
MPI	Multipoint Inoculator
MSDS	Materials Safety Data Sheet
NCTC	National Collection Of Type Cultures (UK)
NGS	Next Generation Sequencing
NHS	National Health Service (UK)
NICE	National Institute for Clinical Excellence (UK)
NPM	Nextera PCR Master Mix
NT	Neutralize Tagment Buffer
^{o}C	Celsius
OED	Oxford English Dictionary
ONT	Oxford Nanopore Technologies
PAL	Pooled Amplicon Library
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PH	Potential of Hydrogen
PHE	Public Health England
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
ppm	Parts Per Million
QC	Quality Control
QRDR	Quinolone Resistance Determining Region
RPM	Revolutions per Minute
RRDR	Rifampicin Resistance Determining Region
RSB	Resuspension Buffer
RSCB	Research Collaboratory for Structural Bioinformatics

RT	Room Temperature
rtPCR	Real time Polymerase Chain Reaction
SBT	Sequence-Based Typing
SCW	Supercomputing Wales
SCAP	Severe Community Acquired Pneumonia
SFARP	So Far As Reasonably Practicable
SG	Serogroup
SPAdes	St. Petersburg genome assembler
SPRI beads	Solid Phase Reversible Immobilization
SQB (Minion)	Sequencing Buffer (Minion)
ST	Sequence Types
TD buffer	Tagmentation DNA Buffer
tRNA	Transfer RNA
TSA	Tryptic Soy Agar
UAT	Urine Antigen Test
UKHSA	United Kingdom Health Security Agency
UTI	Urinary Tract Infection
UV	Ultra-Violet Light
W/V	Weight Per Volume Percentage
WBYE	Washed Buffered Yeast Extract
WGS	Whole Genome Sequencing
WIPO	World Intellectual Property Organisation
XLD	Xylose Lysine Deoxycholate agar

Thesis summary

Legionella causing legionnaires disease is a global health concern affecting thousands of people every year and the treatment requires antibiotics, usually fluoroquinolones or macrolides. As we approach a post antibiotic era, in which common infection treatments and prevention are rendered ineffective, a detailed investigation into the *Legionella* with regard to their susceptibility was lacking. Investigative MIC in *Legionella* required development of a novel media, due to the unsuitability and lack of concordance between currently available media. During this project, LASARUS (*Legionella* Antimicrobial Susceptibility and Universal Screening) media was developed, a clear solid agar lacking the chelating effects of prior solid media. This media was subsequently patented, validated against other MIC methods and published. Using LASARUS, 2,100 isolates from the UKHSA archive were screened for susceptibility against a panel of eight antibiotics, with a wide range of modes of action, to determine a baseline of susceptibility from which ECOFF (Epidemiological Cut Off) values could be determined. Once a base line of susceptibility was available it then became possible to select for resistant isolates and the mechanism of resistance. This screening found very few cases of resistance and where it was present, usually to a macrolide, mediated by *lpeAB* an efflux pump. A dual beta-lactamase (*loxA* and *bla-$_{oxa29}$*) that in combination counter-intuitively reduced resistance to ampicillin, was also found. This project led to the publication of an international position paper, which delineates for the first time the guidance for antimicrobial susceptibility testing in *Legionella* spp. with a specific focus on normalising the methodologies to correlate to the gold standard of broth microdilution (BMD) and for cessation of use of methods, which fail to correlate with BMD.

1 Introduction

1.1 Legionella a historical introduction

1.1.1 The legion

A legion is the Anglo-Normanisation of the Italian word *legiō* used to describe the largest single unit of the Roman empire representing a group of between 3-6,000 Roman infantry men often combined with cavalry (Gibbon 1782; OED 2021), first formed in the early regal period of the Roman empire around 500BC, with some formations surviving well into the 4th Century AD. These units represented an empire which heralded dawn of modern civilisation (Gibbon 1782; Hamblin 1996). This terminology has been carried through modern lexicography for the last 2,000 years and applied to many continental European military groups. A term ubiquitously used by both Shakespeare (Shakespeare 1607; OED 2021) and the daily press. The term was used at the founding of the French Foreign Legion to denote a branch of the French army whose membership was not limited to French nationals in 1831 (Porch 2010). The name for the American legion was likely derived from this group at its founding at the 'Place de la Concorde' in Paris by the victorious American forces in 1919. Since its foundations, the American Legion has been heavily involved in its primary focus of American veteran's affairs, supporting hospitals, and lobbying for the benefits of its members. As an entity with 2.3 million members as of June 2019 they have hosted an annual conference event at conference centres around the US, since its founding.

1.1.2 1976

1976 was an important year for the USA commemorating the bicentennial of the signing of the declaration of independence at Independence Hall in Philadelphia Pennsylvania on the 4th of July 1776. This anniversary event included visits from foreign heads of state including Her Majesty Queen Elizabeth the Second, who toured on HMS Britannia around the US including Philadelphia, (It was only thanks to HMS Britannia that the Queen wasn't staying at the Bellevue-Strand). Due to the military nature of the bicentennial the 58th annual three-day conference attended by around 2,000 people of the American Legion was held concurrently in Philadelphia between the 21st and 24th of July at the Bellevue-Strand hotel, the starting point of human understanding of *Legionella*.

1.1.3 The outbreak

Three days (27/07/1976) after the convention, an American Legionnaire died of an apparent heart attack and a further three days (30/07/1976) later, four subsequent Legionnaires also died of apparent heart attacks. No links were drawn between these deaths and the Bellevue-Strand hotel until a Dr Ernest Campbell realised that three of his patients had attended the convention. On the 31st of July 1976 the American Legions states' adjunct was informed of ten deaths all linked to the convention. Shortly after this the Philadelphia health authorities got involved and by the 16th of August, 182 people had been diagnosed with the unexplained pneumonia and there had been 29 deaths. This triggered wide-spread public concern, partially linked to fears of an influenza epidemic that year, and the clinical presentation similarities between the

outbreak and an influenza outbreak. By this point new infections had decreased and epidemiological investigations into the cause were ongoing (McDade et al. 1977).

1.1.4 The hunt

Influenza viruses were eliminated as a potential cause due to the failure in an agglutination test (McDade et al. 1977). The CDC and others were by this point attempting to elucidate the cause of this disease. Other organisms that were suspected and excluded include ornithosis (*Chlamydia psittaci*), typhoid (*Salmonella typhi/paratyphi* (A, B and C)), the plague (*Yersinia pestis*), tularaemia, and viral haemorrhagic fevers, such as Lassa or Marburg were considered and ruled out. Through electron microscopy of ten autopsied tissue samples, they were able to identify similar bacteria in each of the tested lungs. In this way they discovered the causative agent was bacterial (McDade et al. 1977), fluorescent antibody staining failed to detect the presence of any known microbial species. In an attempt to isolate the bacterium, which failed in mouse models, a guinea pig model was used in which they were inoculated with agents isolated from four lung biopsies. This induced mortality in the animals between 4-6 days and bacilli were found in the liver, lung, and spleen. Suspensions of these organs were then introduced into the yolk sac of chicken eggs. Smears taken from the egg embryos which died showed the presence of a Gram-negative bacillus, 0.3-0.4µm width and 2-3µm breadth as seen in Figure 1.1 (below), the earliest picture of *Legionella pneumophila* (unnamed at this point) (McDade et al. 1977).

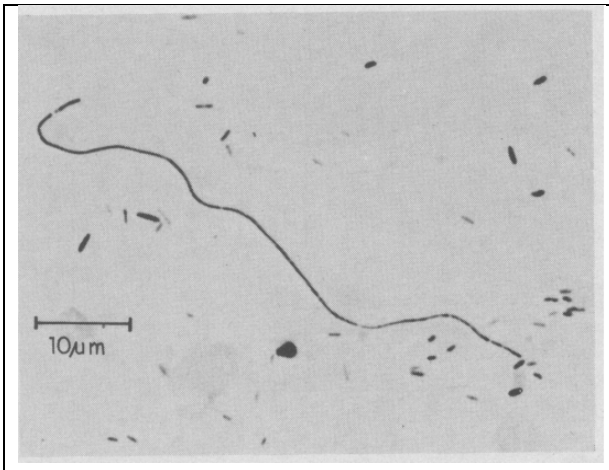


Figure 1.1 The first picture of *Legionella pneumophila*
A photomicrograph of the causative agent of Legionnaires' disease in yolk sac stained by the Gimenez method (McDade et al. 1977).

As well as an investigation into the causative agent, the mode of transmission was also heavily investigated. This included investigation of person-to-person, food, tobacco, alcohol, water, ice, fomites and air source transmission (McDade et al. 1977). Person-to-person transmission was excluded as none of the families of the cases became ill. Equally, a lack of case clustering in hotel rooms was seen. Food transmission was excluded. In total, 28 restaurants were investigated and none were found to be linked to outbreaks, nor were any food sources implicated. Tobacco and ice were also excluded in transmission due to lack of significant association, as well as fomites and zoonotic transmission. No victims reported insect bites or contact with animals or birds. Airborne transmission was hard to prove but showed consistency to observed outbreaks especially with patients with only transient exposure to the Bellevue-Strand hotel (McDade et al. 1977).

1.1.5 Pontiac fever

Similar outbreaks with an unknown aetiology, were retrospectively identified as having been caused by *Legionella*, the first of these was an outbreak of Pontiac fever (a milder form of full-blown LD) in 1968 in Pontiac, Michigan (from which it derived its name, as per the conventions of the time) (Winn 1988). A similar outbreak in 1965 at a DC hospital which infected 78 people with a mortality of 20% (16/78). As well as an outbreak at the also in the Bellevue-Strand hotel in 1974 in which 20 people were infected and two died (Terranova et al. 1978).

1.2 Legionella: modern

Table 2 Taxonomic categorisation of *Legionella pneumophila*

Rank	
Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Legionellales
family	Legionellaceae
Genus	*Legionella*
Species	*Legionella pneumophila*
Subspecies	*Legionella pneumophila* subsp. *pneumophila*

Bacterial classification has developed hugely since Leeuwenhoek first described "his little people" in the first true microbiology paper, published in 1677, (Leeuwenhoek 1677). Since then, a human drive to categorise, list and explain has driven many great scientists to take up and continue the legacy created by Leeuwenhoek in describing and cataloging the macro and microscopic. In Ernest Haeckels' tree of life, 1866, he categorises life into three branches; plant, animal and protista. Protista at this time contained all microorganisms including fungi, algae and bacteria. This classification is shown in Figure 1.2 (Haeckel 1866).

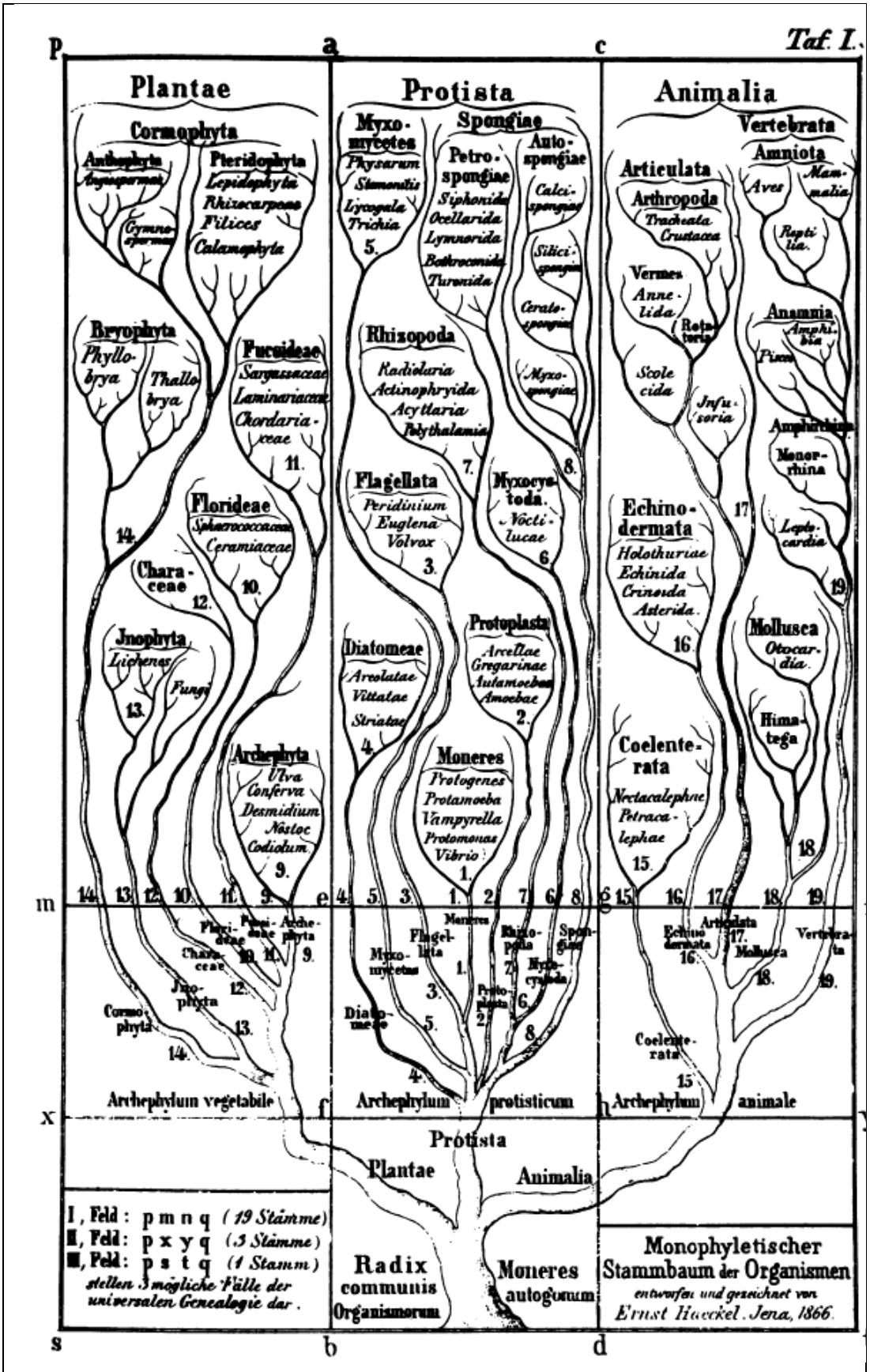


Figure 1.2 Haeckels phylogenetic tree

Showing Haeckels phylogenetic tree 1866 (Haeckel 1866; Woese 1987), highlighting at the time the three orders of life.

Moving forward to Dr Carl Woese a recipient of the Leeuwenhoek medal (one of the highest honours in microbiology). Woese categorised proteobacteria, as a single group named “purple bacteria and their relatives” in 1987 as can be seen in Figure 1.3, due to a universal common ancestor capable of purple photosynthesis, categorised as Eubacteria between cyanobacteria and the Gram-positive bacteria (Woese 1987). The work was based entirely on variations in rRNA structure Figure 1.3.

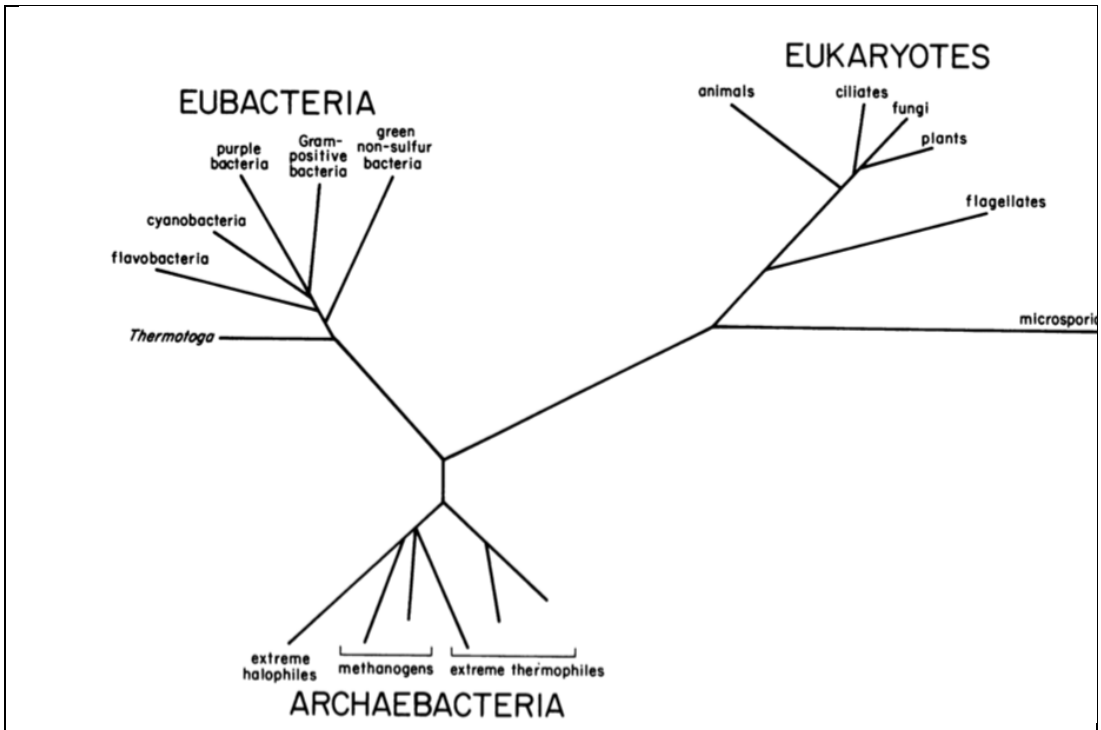


Figure 1.3 A universal phylogenetic tree
A universal phylogenetic tree from rRNA sequence comparisons in 1987 (Woese 1987).

The group of purple bacteria were renamed after Proteus, the Greek God of many forms (Stackebrandt et al. 1988). Using the same rRNA methodology, the Proteobacteria were further sub-categorised into four mains groups, *alpha, beta, delta, and gamma* Figure 1.4 (below).

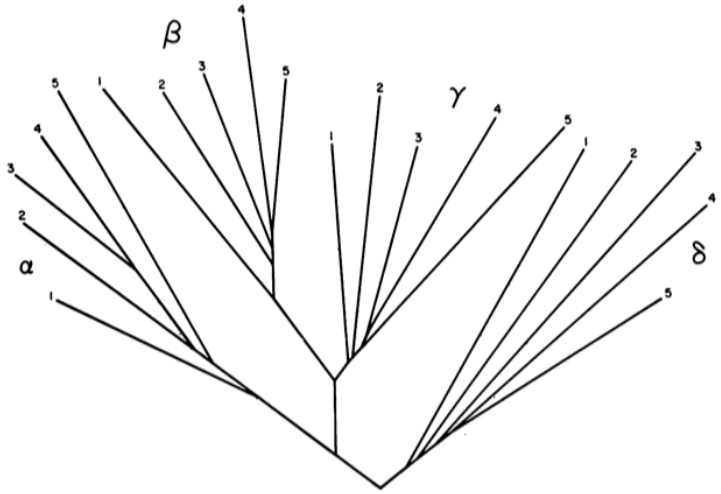


Figure 1.4 Showing the Phylogenetic tree for the purple bacteria Protobacteria

Based on 16S rRNA sequences from (Woese 1987), dividing the group into four subgroups *alpha, beta, delta and gamma.* Of note here is within the gamma section number 2 is *Legionella pneumophila* and number 3 is *Pseudomonas aeruginosa.*

Gammaproteobacteria, are the most genetically diverse of all the groups. To help demonstrate this class' diversity it is worth considering the enormous quantity of virulent human pathogenic species, (*E. coli, Y. pestis, Salmonella, Pseudomonas* spp. *etc)*. Gammaproteobacteria also encompass around 25% of the bacteria found in deep sea sedimentations (Bienhold et al. 2016), and those found universally in soil samples (Kim et al. 2014). They are classed together based solely on their 16S rRNA gene phylogeny (Williams et al. 2010) and grouped as can be seen in the Figure 1.5 (Brenner et al. 2005) further highlighting the breadth of this group.

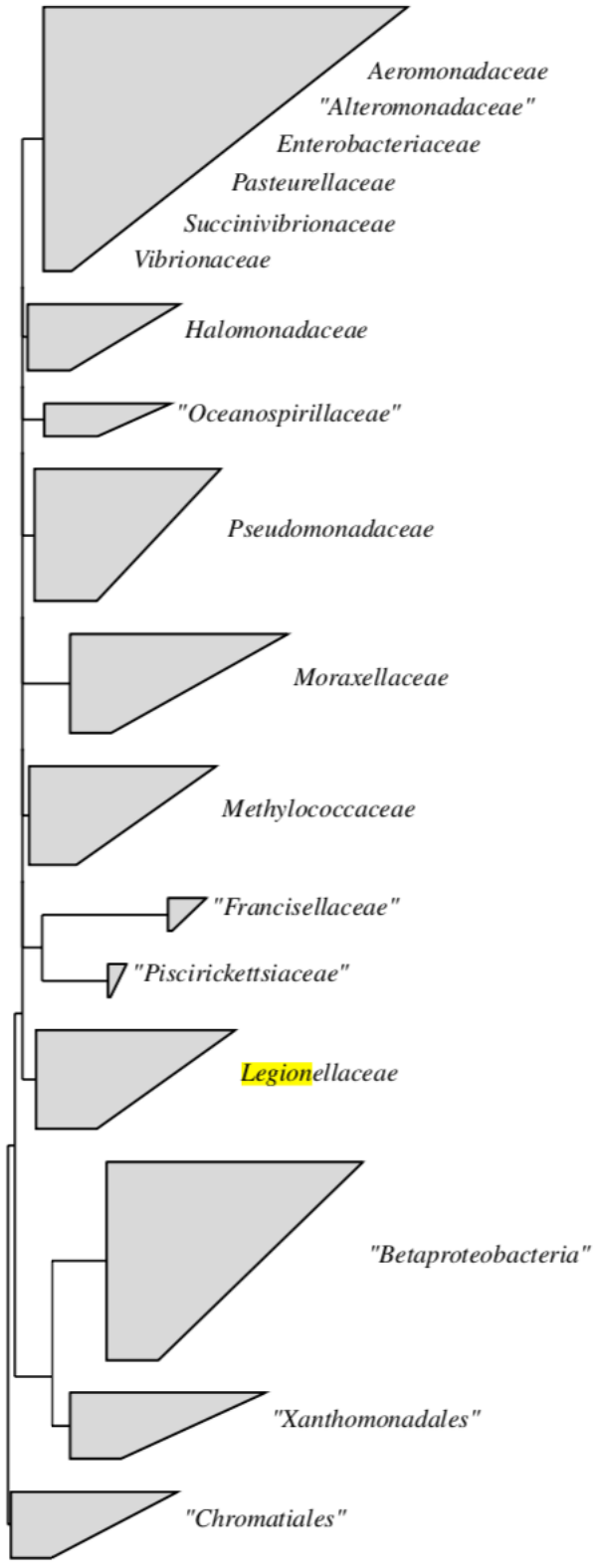


Figure 1.5 Relatedness through 16S rRNA

16S rRNA gene analysis of within Gammaproteobacteria, highlighted is the Legionellaceae group (Brenner et al. 2005).

Within the Legionellae, an order of bacteria within the aforementioned class of the gamma-proteobacteria, Legionellae are all aerobic bacilli. Legionellae contain five distinct genera *Aquicella, Coxiella, Diplorickettsia, Legionella and Rickettsiella*, and these are all linked by four conserved signature indels (CSI) molecular markers consistent with all Legionellae as shown in the Figure 1.6 below.

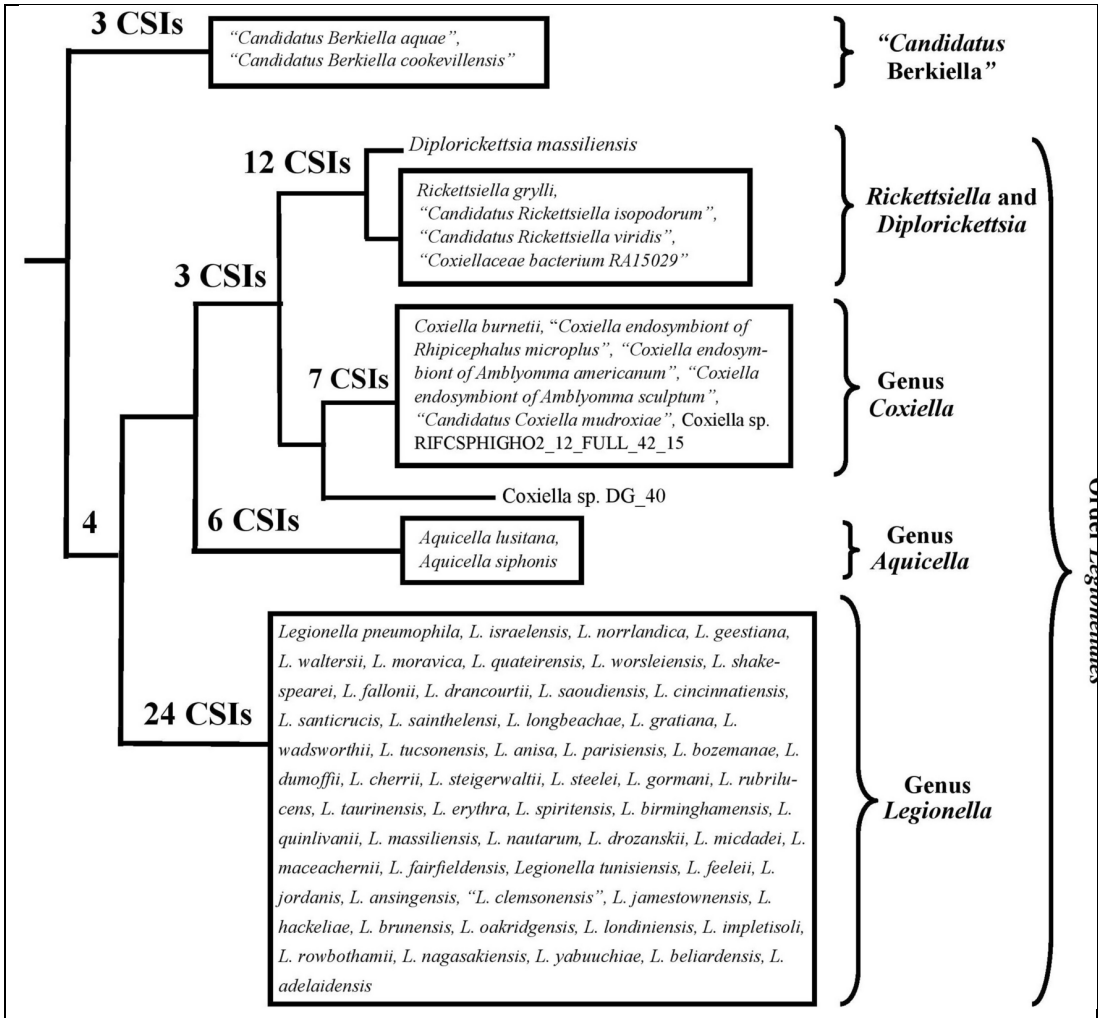


Figure 1.6 Evolutionary relationships among the Legionellales
Taken from (Saini and Gupta 2021) shows the evolutionary relationships among the Legionellales species based on phylogenomic studies, amino acid insertion similarity matrix and the CSIs that are specifically shared by members of different clades within the order Legionellales. The numbers indicated on different nodes represent the total number of identified CSIs that are specifically shared by species from these genera/clades.

The species group together into the pre-existing genus categorisation; *Coxiella, Rickettsia* and *Legionella* the number of CSI for each is 7, 12 and 24 respectively (see

Figure 1.6). Focusing on the *Legionella*, there are 24 CSI linking the entire group. One of the CSIs is linked exclusively to the genus *Legionella* is depicted in a genome alignment in Figure 1.7 below, showing a five amino acid insertion into an endoprotease precursor.

This form of categorisation requires NGS (Next Generation Sequencing) and does not involve the function of a gene, location, morphology, functionality or virulence of a bacterium but categorises them based on shared and unique patterns in their sequences, and does so very with a high degree of power (Saini and Gupta 2021).

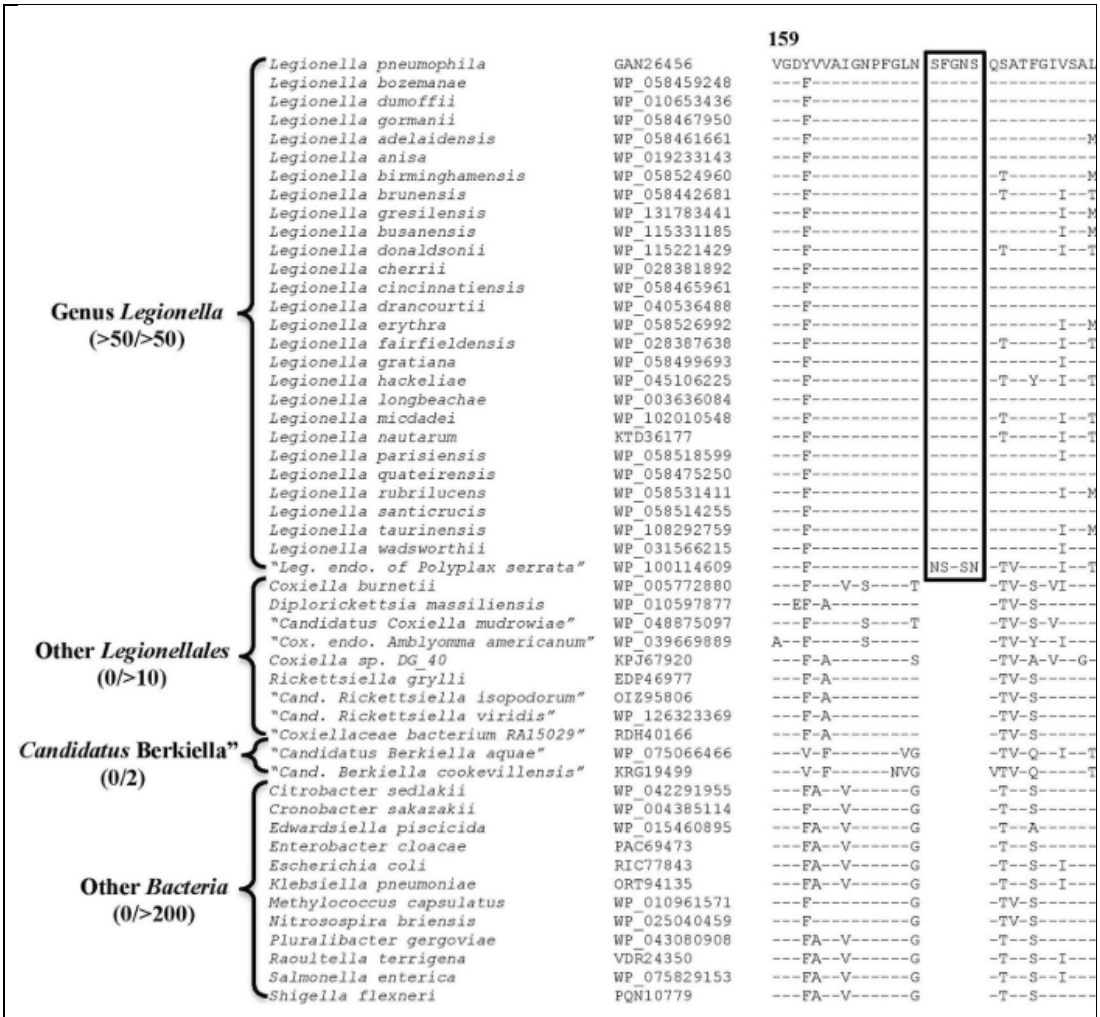


Figure 1.7 Sequence alignment of a periplasmic serine endoprotease

A sequence alignment of a periplasmic serine endoprotease DegP precursor and boxed shows a five amino acid insertion which is conserved across all *Legionella* species but not *Legionella* les or other bacteria (Saini and Gupta 2021).

1.3 Legionella species

As of 2021, there are currently 63 *Legionella* species that are recognised as validated by the German Type strain collection (DSMZ) https://lpsn.dsmz.de/genus/*Legionella.* Furthermore, there are an additional five species, which are in the process of being approved. Of these species few have consistently been linked to disease in humans apart from *L. pneumophila* and *L. longbeachae.* The few others that have been linked to human disease sporadically in non-(severely) immunocompromised patients are displayed in the Table 3 below:

Table 3 *Legionella* species linked to human disease

Legionella species	Serogroups	Causes human illness	
L. anisa		++	Sporadic and an outbreak of Pontiac fever
L. bozemanii	2	++	Sporadic and nosocomial cases
L. cardiaca		+	
L. cincinnatiensis		+	
L. clemsonensis		+	Green fluorescing *Legionella*
L. dumoffii		+	Sporadic cases and a single documented outbreak
L. feeleii	2	+	
L. gormanii		+	
L. hackeliae	2	+	
L. jordanis		+	
L. lansingensis		+	
L. longbeachae	2	++	
L. lytica		+	
L. maceachernii		+	
L. micdadei	Tatlock	+	
L. oakridgensis		+	
L. pneumophila	15	++ +	
L. sainthelensi	2	+	
L. steelei		+	
L. tucsonensis		+	

1.4 Hosts

All Legionellae are intracellular parasites, predominantly of amoebal species such as: Hartmanella or *Acanthamoeba*, and can also thrive in invertebrates and mammals (Palusińska-Szysz and Cendrowska-Pinkosz 2009). The diversity of these hosts gives *Legionella* an enormous breadth of host covering such a wide environment that they are found ubiquitously.

Due predominantly to its parasitic nature which in its normal environment has led to an abundance of readily available amino acids which Legionellae use as a direct food source. For this reason, Legionellae have lost the ability to create several amino acids themselves, with the most significant being L-cysteine but also includes arginine, isoleucine, leucine, threonine, valine, methionine, phenylalanine, tyrosine, and serine (George et al. 1980; Taylor et al. 2009).

Amoebal and protozoan species often survive well within artificial fresh water systems (air conditioning units, potable water systems, cooling towers, but really anywhere fresh water is stored in a closed system). These are the primary source of human exposure (Taylor et al. 2009). On top of this advantage *Legionella* can persistent well in a biofilm, both as intracellular parasites and free-living species within the extra cellular matrix of the biofilm. The presence of a biofilm host enhances the survivability of the *Legionella* species as they are more resistant to extreme heat or biocide treatment (Taylor et al. 2009; Shaheen et al. 2019).

Humans are considered a dead-end host, as there is only one report of *Legionella* person to person in Portugal in 2014 (Borges et al. 2016). Anecdotal reports from an

individual working on the investigation noted that the carer may have acquired *Legionella* from the index patient as they were providing end of life nursing care, in a non-ventilated room, and were most likely to have inhaled *Legionella* produced by coughing from the index case (Borges et al. 2016).

1.4.1 Prevention/Disinfection

The UK Health and Safety Executive (HSE) has published a document (L8) (https://www.hse.gov.uk/pubns/books/l8.htm) (UKHSA 2013) that describes the approved code of practice and guidance on regulations for Legionnaires' disease and the control of *Legionella* bacteria in water systems. The health and safety aspect of this refers almost exclusively to *Legionella pneumophila* as the major risk of infection; however, cfu/L quantification is recommended to be carried out for all *Legionella* species. This book summarises the legal requirements for those responsible for *Legionella* control as seen in Figure 1.8 and also suggests ways in which *Legionella* control should be approached and managed.

Information box: Summary of the HSW Act, sections 2, 3 and 4

Section 2 places a duty on employers to ensure the health, safety and welfare o employees so far as reasonably practicable (SFARP). More guidance on the principles of SFARP may be found on the HSE website (www.hse.gov.uk/risk/ theory/alarp1.htm). Section 2 also requires employers to consult with trade union safety representatives on matters affecting health and safety in the workplace. Employers of more than five people must also prepare a written health and safety policy and bring it to the attention of employees.

Section 3 requires employers to ensure that non-employees who may be affected by work activities are not exposed to risks to their health and safety.

Section 4 places a duty on anyone responsible for the workplace to ensure that the premises, plant and machinery do not endanger the people using them.

Figure 1.8 An exert from HSE L8 summarising HSW act 2,3&4
Attached a short extract to highlight the key points of this document.

A publication by Sarjomaa, *et al.*, outlined methods by which LD can be prevented in hospitals. They suggested five methods: chlorination, heat treatment, point of use filtration and silver and copper ions disinfection of water supplies (Sarjomaa et al. 2011). A 4-year assessment on *Legionella* treatment using chlorination showed that chlorination is effective in reducing the concentration of *Legionella* to acceptable levels but when regular treatment stops the concentration can increase, further emphasising the continual need for water treatment (Springston and Yocavitch 2017; Vincenti et al. 2019; Carlson et al. 2020).

Point of use filtration devices which fit to taps are available but expensive and are not long lasting and have been shown to slough off *Legionella* found growing in the filters. (Springston and Yocavitch 2017; Carlson et al. 2020).

Heating and flushing remain a preferred method as its cheap and easy to implement, it is important that the water system reaches 70ºC to rapidly eradicate the *Legionella* however it too has problems and fails to completely eradicate *Legionella* when it is ingrained within a biofilm or in a system with high calcium build-up and needs to be

carried out regularly (Mouchtouri et al. 2007; UKHSA 2013; Springston and Yocavitch 2017; Carlson et al. 2020).

UV light successfully eradicates *Legionella* but has some disadvantages including high energy cost and limited longevity, which means that it is used infrequently, with the emergence of UV-LEDs this method may become more popular as the price reduces(Vilhunen et al. 2009; Carlson et al. 2020).

Copper and silver ions have also been used for the removal of *L. pneumophila* from water systems. Liu, *et al.* found that concentrations of copper and silver above 0.4 and 0.04ppm respectively resulted in a significant decrease in the bacteria (Liu et al. 1994). Rohr, *et al.* also found that copper and silver both had a disinfectant effect, silver more so than copper. They used 800µg/mL copper and 80µg/mL silver in combination, and found that it caused a 5-log decrease in the amount of *L. pneumophila* (Rohr et al. 1996) similar work has been shown in 2011 in a hospital setting (Lin et al. 2011; Carlson et al. 2020). A 2002 review by Kim, *et al.* investigated published literature regarding the efficacy of various disinfectants against *Legionella* in water systems. The disinfectants investigated were metal ions, oxidising agents (including chlorine), non-oxidising agents and UV light. The overarching finding was that oxidising agents were more effective at eradicating the bacteria from water than non-oxidising agents. The most effective oxidising and non-oxidising agents were chlorine and 2,2-dibromo-3-nitropropionamide respectively (Kim et al. 2002).

An example of the difficulties in the removal and management of *Legionella* from a water system is the investigation into the multiple species of *Legionella* within the water system of a large occupational building. This showed that the building had been colonised for over 30 years with three distinct STs (27, 68 and 87) however, no

associated disease cases had been reported and all attempts to remove them long term failed (David et al. 2018).

The crucial factor that must be acknowledged when selecting a method of water treatment is the implications it may have when on human consumption in potable water systems. Metals such as copper and silver can be poisonous in excess, and the same is true for chlorine; all of which are methods of choice for *Legionella* control (Sarjomaa et al. 2011).

1.4.2 Legionella longbeachae

Legionella longbeachae occupies a different environmental niche as compared to most other *Legionella* species. Instead of colonising a water system it is found predominantly in potting soil and other rotting vegetation, such as bark and sawdust (Whiley and Bentham 2011). This species is the primary cause of LD in Australia and New Zealand, due to their potting soil containing a high concentration of infected pine bark. Methods of alleviating disease related to *L. longbeachae* include leaving soil to rest after buying, as well as using gloves and a face mask when handling potting soil (O'Connor et al. 2007; Mohammadi et al. 2021). An example of the associated health warnings on packaging for horticultural growth medium are shown in Figure 1.9.

Figure 1.9 Health warning on Australian potting mix
A mock-up label found on all potting mixes warning of the risks of *L. longbeachae* within potting mixture in Australia and New Zealand.

1.5 L. pneumophila serogroup 1

L. pneumophila serogroup 1 (Lp Sg 1) is the most common serogroup causing disease. A 2002 Europe-wide study by Helbig, *et al.*, analysed 1,355 strains of *L. pneumophila* obtained from LD cases. They found that of the strains tested, 78.5% were Lp Sg 1. The remaining 21.5% of the strains were other serogroups of *L. pneumophila* (Helbig et al. 2002; Cunha et al. 2016). A caveat of this study was the inherent bias in diagnostics, which artificially selects for serogroup 1.

A 2009 study by Harrison, *et al.*, analysed 167 clinical *L. pneumophila* isolates from England and Wales, of which 97.6% were found to be Lp Sg 1. Interestingly, they compared this to 276 environmental isolates, of which only 55.8% were Lp Sg 1. Each of the clinical and environment isolates were analysed using sequence-based typing (SBT). Comparison of the sequence types discovered little overlap between human and environmental populations (P=<0.0001), indicating a potential for differences in infectivity and pathogenicity (Harrison et al. 2009).

1.6 Legionnaires' disease cases

Legionnaires' disease and the less severe Pontiac fever happens throughout the world. Data from UKHSA (UK Health Security Agency) formally: Public Health England (PHE) reported a monthly breakdown comparing 2020 to the previous three years Figure 1.10. This shows more cases are seen during the hotter months of the year (June, July, August & September). This is due, partly to travel related cases, and increased use of hotels and holiday accommodation, spa pools and air conditioning

units. In 2017 there was a spike in travel associated cases from an outbreak of ST616 from Dubai (Dabrera et al. 2017).

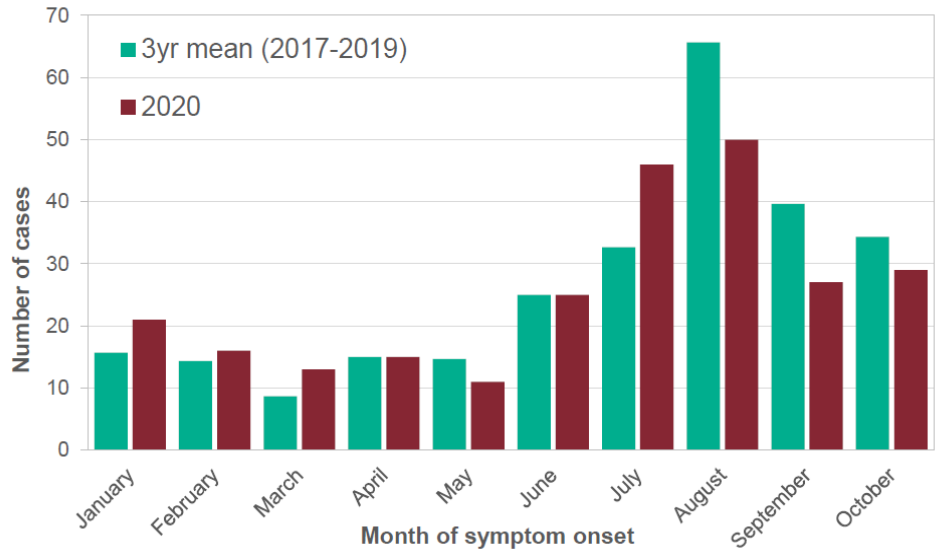


Figure 1.10 Cases of Legionnaires disease
Confirmed cases of Legionnaires' disease by month of onset in 2020 compared to the mean of the previous three years, as reported annually by UKHSA (UKHSA 2020).

In Europe, the case rate for LD is around 2.2 cases per 100,000 and there has been a gradual year on year increase in reports, as can be seen in Figure 1.11 and more probably a due to increased detection and testing and improvements rather than increasing case numbers.

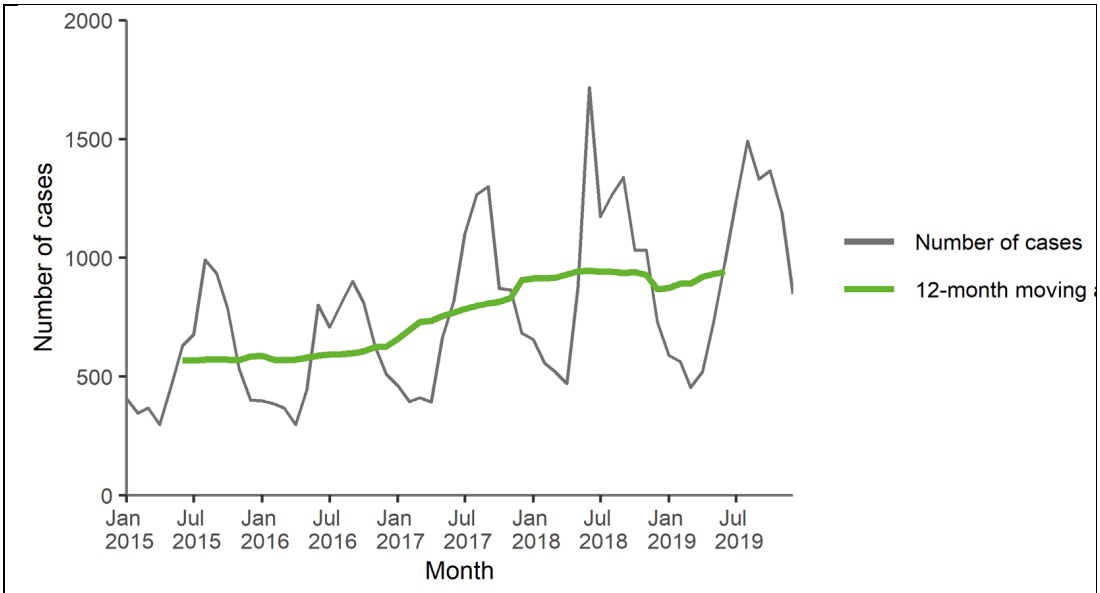


Figure 1.11 Distribution of Legionnaires' disease cases by month

Distribution of Legionnaires' disease cases by month, EU/EEA, 2015–2019 showing the seasonal fluctuations as well as the 12 month rolling average (ECDC 2019).

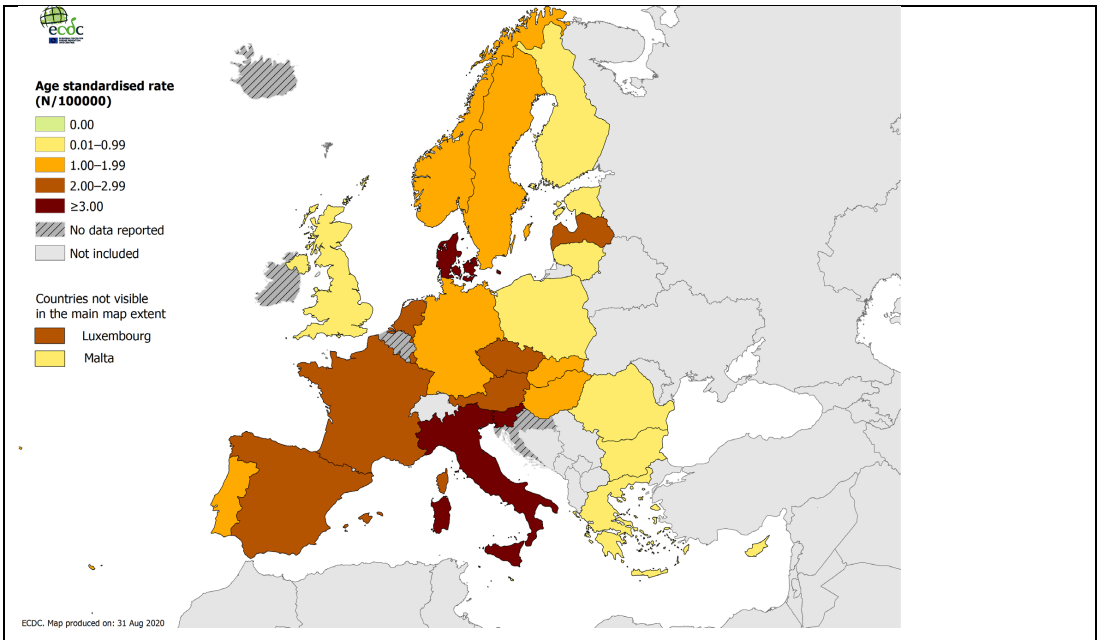


Figure 1.12 European distribution of Legionnaires' disease

Distribution of Legionnaires' disease cases per 100 000 population by country, EU/EEA, 2019 (ECDC 2019).

Serogroup distribution in clinical cases is conserved across Europe, where most countries mirror France with 95% of disease caused by *Legionella pneumophila* serogroup 1 (Doleans et al. 2004), as can be seen in Table 4 (modified from the ECDC,

2019 report). There are exceptions which skew this data; Denmark has a *Legionella pneumophila* serogroup 1 distribution of only 60% (n=67/111), and a large proportion (22%) of Lp Sg 3 (n=25/111) (Statens Serum Institut 2020). The abnormally high rates of Lp Sg 3 in Denmark are apparent as they contributed to 71% of Lp Sg 3 cases in Europe in 2019. This could be attributed to bias in the testing systems and models employed for surveillance between countries. The majority of countries rely on diagnostics that are heavily biased for *Legionella pneumophila* serogroup 1 detection (e.g. UAT), whereas Denmark has a more comprehensive PCR and culture-based system, capable of detecting non-serogroup 1 cases (Mercante and Winchell 2015).

Table 4 A table showing European distribution of *Legionella pneumophila* serogroups

Modified from ECDC annual *Legionella* report (ECDC 2019).

Serogroup (SG)	Number	%
1	923	83
2	9	<1
3	35	3
4	2	<1
5	8	<1
6	17	2
7	5	<1
8	6	<1
9	1	<1
10	9	<1
11	0	-
12	0	-
13	1	<1
14	1	<1
15	3	<1
L. pneumophila non serogroup 1	7	<1
L. pneumophila serogroup mixed	3	<1
L. pneumophila serogroup unknown	76	7

1.7 Outbreaks

L. pneumophila causes disease through the inhalation of an aerosolised bacteria. These aerosols often arise in cooling towers, home water systems and air conditioning units. Human to human transmission of the disease is rare having only been reported once (Carrington 1979; Bartram et al. 2007; Cunha et al. 2016).

Once inhaled, the bacteria invade the lower lung, reaching the alveoli where they are engulfed by macrophages (Schaechter 2009). The bacteria begin to grow and divide within membrane-bound vacuoles to avoid degradation. The bacteria also inhibit phagosome-lysosome fusion (Carrington 1979). This is partially accomplished by the well-studied Dot/icm secretion system virulence factor, a type IV secretions systems which can deliver over 300 proteins directly into the cytoplasm of the affected host (Durie et al. 2020).

Infection by *L. pneumophila* (known as legionellosis) can cause one of two conditions: Legionnaires' disease (LD) or Pontiac fever (PF) (Fields et al. 2002; Cunha et al. 2016). The main symptoms of LD are fever, myalgia, headache, shortness of breath, a cough, which may either be dry or productive, tachycardia, sweating, or shivering and loss of appetite. Pontiac fever is a much milder form of LD and the symptoms are similar, but much less severe, and without pneumonia (Tossa et al. 2006). A person with PF can be expected to make a full recovery within two to five days without medical or pharmacological intervention. However, if left untreated, a person with LD may deteriorate into rapidly progressive pneumonia, multi-organ failure, coma and death (Fields et al. 2002; Demello et al. 2007; Cunha et al. 2016).

While *Legionella* infections are typically sporadic, predominantly with single individuals affected, the disease is renowned for causing outbreaks, which create huge

media attention. Listed in Table 5 are some of the most well publicised outbreaks and their key features.

Table 5 Infamous outbreaks of Legionnaires disease

City, Country	Year	Case #	Fatalities (%)	Source	Key features
Philadelphia, USA	1976	182	16	Hotel air conditioning	First documented outbreak
Stafford, UK	1985	68	32.4	Air conditioning plant	Nosocomial outbreak
London, UK	1988	70	4.3	Cooling towers	Cooling towers in central London
Cruise ships,	1994	50	2	Whirlpool spa	Nine cruises Apr-June, 1994
Transplant centre, USA	1996	25	48	Hospital water	Transplant centre
Murcia, Spain	2001	800	1	Cooling Tower	Largest reported outbreak
Barrow-in-Furness, UK	2002	179	3.9	Cooling tower	
Cherokee, USA	2004	7	43	Cooling tower	Affected a care home
South Dakota, USA	2004	14	7	Water fountain	
South Wales, UK	2010	24	7	Cooling Tower	
Stoke-on-Trent, UK	2012	21	9.5	Whirlpool spa	Whirlpool-spa on display at shop
Edinburgh, UK	2012	92	4.5	Cooling tower	Largest UK outbreak
Lisbon, Portugal	2014	377	4	Cooling tower	
New York, USA	2015	138	11.6	Cooling tower	Changed USA policy on testing
Brescia, Italy	2018	33	6	Chiese River	Lp Sg2

The first known widespread outbreak of LD was at the Bellevue-Stratford hotel in Philadelphia in July 1976 (Tsai et al. 1979), as explained in the historical introduction, and this was then followed by further globally recognised outbreaks. In the UK, Stafford was the first UK reported hospital outbreak.

The case fatality rate and mortality of LD varies depending on a number of factors. These include: the severity of the infection (which strain of *Legionella*), the time taken for antibiotic treatment to commence and patient demographics (age, disease and

transplant status), as well as level of exposure, proximity to source, water droplet size and degree of lung penetration (Tkatch et al. 1998; Bartram et al. 2007).

Within outbreaks it is important to note that the number of identified cases and the number of actual cases are not necessarily or indeed likely to be very similar. As such this massively skews the case fatality data. An example of this is the Murcia, Spain outbreak of 2001 with 800 cases reported and six deaths. The outbreak was linked to cooling the towers in six buildings one of which was a hospital (Ana et al. 2003). This outbreak triggered an enormous screening program detecting many people, who for whatever reason presented asymptomatically or with mild symptoms being recorded as having LD whereas in fact they should have been recorded as PF positive of *Legionella* positive but not LD positive. This led to the artificially reduced case fatality rate of 1%.

On the other end of the scale, the 1996 outbreak in a transplant centre reported a case fatality rate of 48% (Kool et al. 1998). Between these two extremes it is impossible to separate bacterial virulence factors from patient susceptibility influences; for all bacterial infections immunocompromised status vs healthy immunocompetent populations are well identified as contributing to outcome.

For this reason, mortality (or at least reported mortality) of the disease can vary considerably between outbreaks. The original 1976 outbreak in Philadelphia had a case fatality rate of 15.4%, and in the largest outbreak in Murcia in 2001, it was just 0.8% (Tsai et al. 1979; Ana et al. 2003). The World Health Organization estimates that the mortality of hospitalised cases is around 15-20%, but up to 40% for nosocomial cases (Bartram et al. 2007; Fastl et al. 2020).

The majority (all those reported in Table 5 apart from one) were *Legionella pneumophila* Sg1. However, the Chiese river outbreak in Italy was caused by *L. pneumophila* Sg2 (Scaturro et al. 2021). All the patients infected were urine antigen test negative, highlighting the importance of detection of non-sg1 isolates and the global need for less biased screening tests.

1.8 Risk factors for Legionnaires' disease.

As with any disease, there are risk factors that increase both the severity of the disease and the likelihood of getting the infection in the first place. For *Legionella* infections common risk factors are: aged over 50, immunocompromised status, chronic illness, smokers or history of chronic alcohol consumption (Cunha et al. 2016). A list of associated risk factors is also detailed in Table 6 taken from the UKHSA *Legionella* annual report data.

Table 6 Underlying medical conditions and risk factors reported in confirmed cases of Legionnaires' disease, 2014 to 2016 UKHSA

CONDITIONS	2014 %	2015 %	2016 %
Any underlying condition	242 (73.1)	286 (74.5)	265 (74.6)
Diabetes	50 (15.1)	67 (17.4)	46 (13.0)
Heart conditions	96 (29.0)	122 (31.8)	101 (28.5)
Immunosuppression*	40 (12.1)	45 (11.7)	46 (13.0)
Liver conditions	12 (3.6)	15 (3.9)	16 (4.5)
Neoplasms	26 (7.9)	28 (7.3)	25 (7.0)
Renal disorders	12 (3.6)	19 (4.9)	13 (3.7)
Respiratory conditions	26 (7.9)	52 (13.5)	43 (12.1)
Smoking	109 (32.9)	110 (28.6)	115 (32.4)

* Immunosuppression due to other conditions or clinical treatments

NB: Individual cases may have reported more than one underlying condition/risk factor. (UKHSA 2016)

1.9 Treatment

1.9.1 Empirical antibiotic usage:

Empirical usage of antibiotics is common with community acquired pneumonia (CAP) and the British Thoracic Society recommends amoxicillin, clarithromycin, or doxycycline for CAP. Hospital inpatient or a patient with moderate to severe CAP are most commonly prescribed amoxicillin in combination with a macrolide. As an alternative, doxycycline or levofloxacin are also recommended. For high severity CAP empirical advice suggests co-amoxiclav in combination with a macrolide. With penicillin concerns regarding allergies, third generation cephalosporins such as cefotaxime with clarithromycin are recommended (Lim et al. 2009; BNF 2021a).

1.9.2 Treatment post diagnosis:

Once a patient has been diagnosed with LD, antibiotic treatment must start immediately, a delay in the onset of treatment is associated with significant increase in mortality (Pedro-Botet and Yu 2009). The British Thoracic Society (Lim et al. 2009) recommends an oral fluoroquinolone (for seven days) as the first line of treatment for mild LD, no treatment is needed for the milder PF, but in severe cases a combination of fluoroquinolones and macrolides can be used for 7-10 days (extended to 21 if needed) (BNF 2021a). There is also some recommendation (though lacks systematic evidence-based validation) for the addition of rifampicin to this antibiotic combination. (Lim et al. 2009). The same study also stated that tetracyclines, tigecycline, other fluoroquinolones and other macrolides (especially clarithromycin) can also be effective.

1.9.3 Diagnostics

Apart from the aforementioned symptoms of LD it can also present with less common symptoms include diarrhoea and confusion or delirium (https://www.hse.gov.uk/legionnaires/symptoms.htm) (HSA 2020). Differentiating this from other typical causes of pneumonia can be done using a urine antigen test, or by PCR and culture of lower respiratory secretions (CDC 2017; Pierre et al. 2017; Ginevra et al. 2020). The urine antigen test is a standard diagnostic tool in any patient suspected to have, or with confirmed, atypical pneumonia, especially those not responding to empiric treatment of CAP (Pierre et al. 2017). However, the urine antigen test (UAT) is not as effective at detecting *Legionella* which are not *L. pneumophila* and predominantly detects Sg1 which accounts for around 80% of LD cases. Therefore, the test may give a false negative in some cases (Couturier et al. 2014). Ideally a *Legionella* species and *Legionella pneumophila* specific PCR followed by culture of a lower respiratory sample should be carried out.

1.10 Culturing Legionella

Legionella are difficult to grow. This is because of the complex nutritional requirements due of their intracellular nature. BCYE-α, a buffered charcoal yeast extract agar is the agar of choice for growing *Legionella* species and contains activated charcoal (Edelstein 1981). This component of the solid agar is essential for absorbing toxic compounds which inhibit *Legionella* growth; however, charcoal will also absorb others such as antibiotics rendering accurate determination of accurate agar-based MICs impossible.

1.11 AMR antibiotics and Legionella

Identification of antibiotic resistance mechanisms using WGS of the Legionellaceae has enabled the detection and characterisation of the emergence of strains with reduced sensitivity. An example of this is the detection of the genes *lpeAB* that encode a macrolide efflux pump (Vandewalle-Capo et al. 2017). This efflux pump has been shown to significantly increase the resistance of strains to azithromycin, as can be seen in Figure 1.13 where the distribution curve shows almost normal distribution but with the presence of a positive skew. On top of this, the graph Figure 1.13 shows that *in vitro Legionella* can be artificially stressed to induce highly resistant strains in a way that is not possible for all bacteria, the black bar in the bar charts. This further highlighting the potential for AMR in *Legionella*.

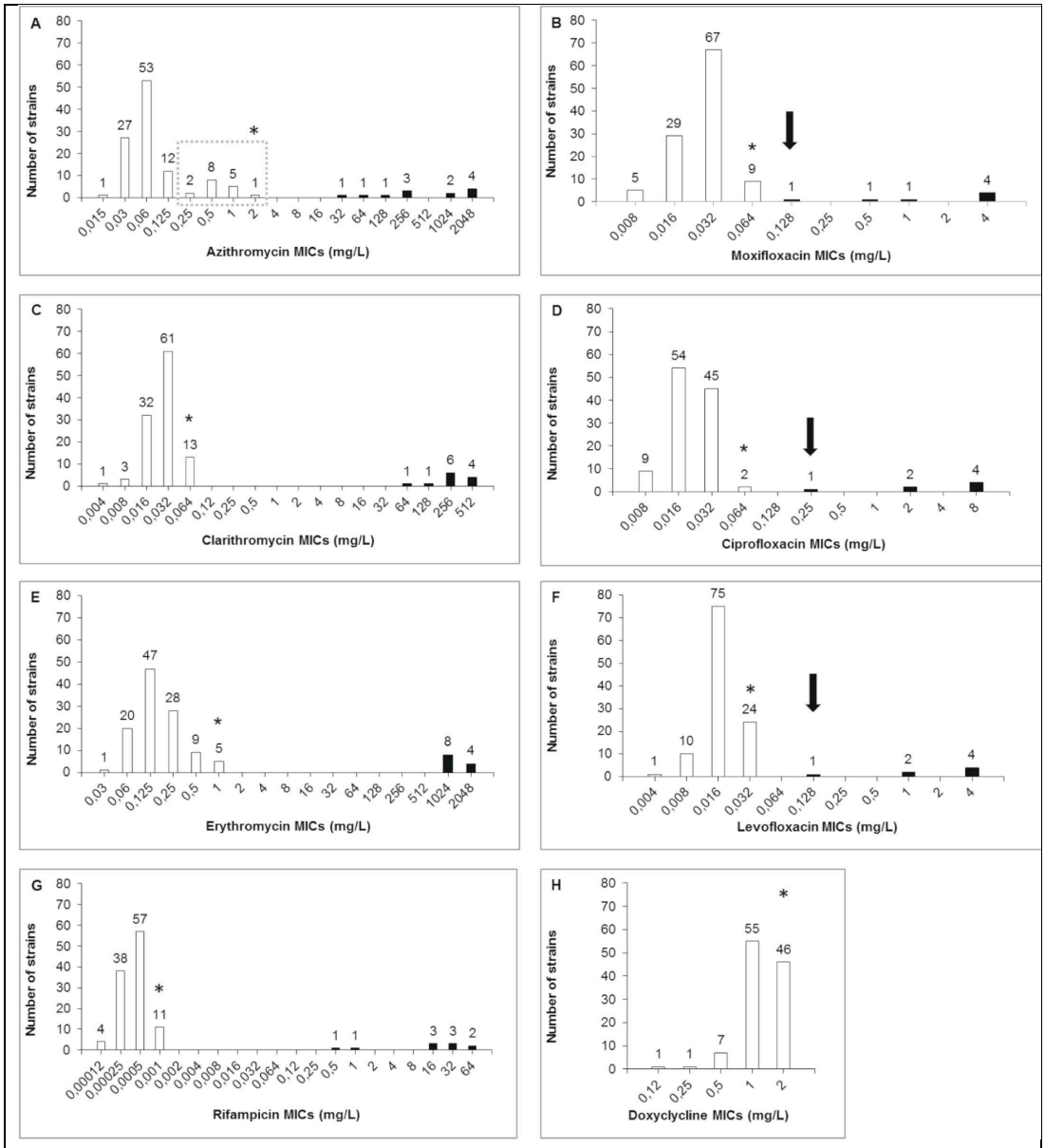


Figure 1.13 Distribution of antibiotic MICs for *Legionella pneumophila*
Legionella pneumophila (n=109) SG1 clinical strains (white bars) and *in vitro*- passaged antibiotic-resistant strains (black bars). Taken from: (Vandewalle-Capo et al. 2017).

Common high-level tetracycline resistance *L. longbeachae* more prevalent than *L. pneumophila* in CAP in Australia and New Zealand, has been found to be associated with a tetracycline destructase and is equally important and further evidence of *Legionella* species developing resistance mutations to clinically relevant antimicrobials (Forsberg et al. 2015).

Levofloxacin resistant isolates have also been shown *in vivo*. The underlying mechanism for fluoroquinolone resistance has been shown to be a *gyr*A83 mutation, located in the GyrA QRDR (Quinolone Resistance Determining Region), potentially leading to treatment failure (Shadoud et al, 2015). Understanding the mechanisms of resistance is particularly important for the Legionellaceae where the slow growing nature of the organism and a delay in correct effective therapy is associated with significant increase in morbidity and mortality (Heath et al. 1996).

Currently, there is no international consensus on antimicrobial susceptibility testing (AST) methodology for systematic investigation of *Legionella* species. A variety of methodologies to determine MICs for target antimicrobials are available. The gold standard method for most clinically relevant bacterial pathogens is broth microdilution (Portal et al. 2021a; Portal et al. 2021b).

International standardization via CLSI (Clinical and Laboratory Standards Institute) EUCAST (European Committee on Antimicrobial Susceptibility Testing) or other reference centres requires several conditions to be met (CLSI 2020) & (EUCAST 2021):

(i) standardization to a defined bacterial inoculum load;

(ii) a defined growth medium (either commercially available from more than one source or constituents fully specified);

(iii) standardized dilutions of defined (relevant) antimicrobials (or defined concentrations for disc diffusion);

(iv) AST to be validated by concurrent testing of universally available (culture collection ATCC(https://www.atcc.org/)-, DSMZ (https://www.dsmz.de/)- and/or NCTC (https://www.culturecollections.org.uk/collections/nctc.aspx) bacterial prototype strains (both defined sensitive and resistant strains).

There are often a number of other surrogate methods (commercial and non-commercial) for AST determination of fastidious and non-fastidious bacterial pathogens; however, these are validated against BMD using internationally accepted reference methods and organisms to attain ISO (International Organization for Standardization) standards. The term 'epidemiological cut-off' (ECOFF) is defined as the *in vitro* MIC threshold that allows the discrimination of wild-type strains from those with acquired resistance mechanisms(Turnidge et al. 2006). To date, there are no ECOFF values appointed for *Legionella* species, due in part to the variation in MIC values when compared with different methodologies.

For *Legionella* species. AST, BMD is time-consuming and difficult to run on multiple isolates due to slower growth rates and complex enriched medium requirements. Solid-based methods have been limited by the required inclusion of activated charcoal to remove growth-inhibitory toxins. The degree of antimicrobial compound adsorption is unknown and likely to be highly variable, with the consequence of elevating the MIC.

Antimicrobials can either be incorporated directly into the solid agar (as a range of dilutions) or applied on poured plates by overlaying commercial antimicrobial gradient strips. The standard Kirby–Bauer disc diffusion method (inhibition zones around paper discs impregnated with antimicrobials) cannot be interpreted without definition of accepted zone diameters for reduced sensitivity (García et al. 2000). Significant discrepancies between MICs determined using BMD methods and BCYE-α have been noted previously. (Ruckdeschel and Dalhoff 1999; Bruin et al. 2012) However, no study has performed a systematic comparison between BMD and BCYE-α media. This limits the currently available data for international reference by

EUCAST, with current data based solely on antimicrobial gradient strips (such as E-tests) on BCYE-α. Before ECOFF values can be established, unification of the existing data in the literature, through cross-comparison of shortfalls and inherent variability relative to the gold standard (BMD) utilized for other bacterial species, is required as the first step to agreeing international guidelines for AST in Legionellaceae

1.12 Gentamicin

Aminoglycosides are a class of antibiotics named for their structural inclusion of an amino-modified sugar core. The first examples derived from the bacteria species *Streptomyces* are all identified by the ending -mycin. The prototype example of the first generation of this class is exemplified by the antibiotic streptomycin, first clinically used in 1945 and discovered in 1943 by Schatz and Waksman (Schatz et al. 1944). Waksman (alone) received the 1952 Nobel Prize in medicine in recognition of this achievement (Wainwright Milton 1991; Krause et al. 2016). Gentamicin (note the lack of -mycin) was discovered in 1963, derived from a *Micromonospora purpurea* (Weinstein et al. 1963). Other environmental bacterial species have yielded other antimicrobials of this class, which has been extended to include multiple variants up until the early 1970s, with the addition of amikacin (Kawaguchi 1976). All aminoglycosides contain the same core structure as can be seen in the Figure 1.14 (Krause et al. 2016).

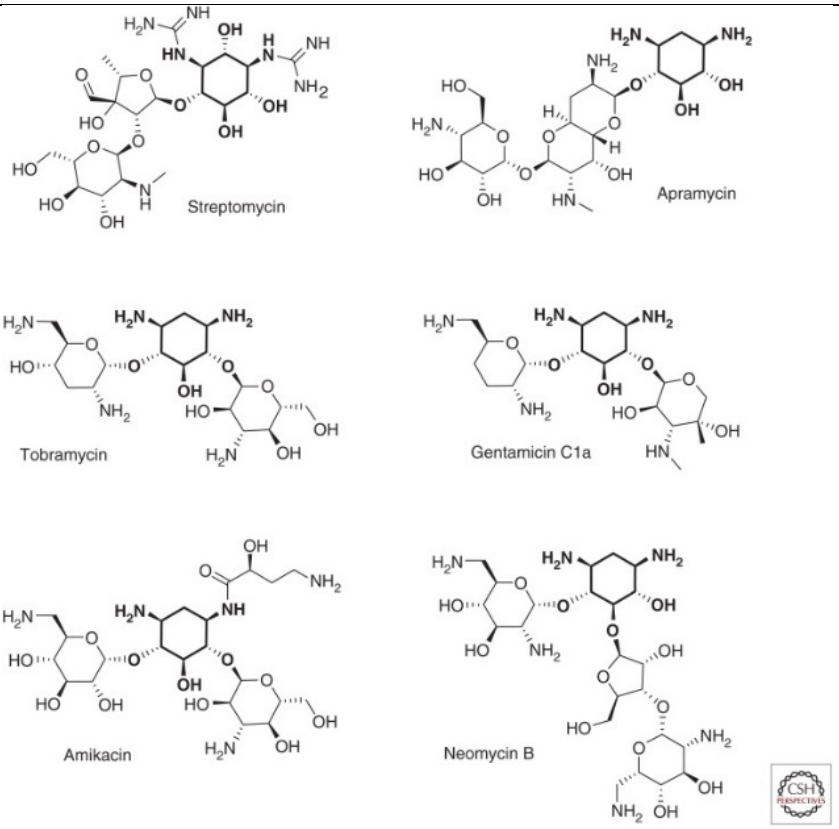


Figure 1.14 Structures of aminoglycosides; streptomycin and apramycin, tobramycin, gentamicin, and amikacin, and neomycin.
Taken from: (Krause et al. 2016).

Aminoglycosides have a broad scope of activity across both Gram-positive and -negative organisms and this is due to their conserved mode of action, which is to inhibit protein synthesis (Krause et al. 2016). Aminoglycosides are absorbed into the cell via active electron transport (respiration), which renders them ineffective on anerobic or facultative anaerobic bacteria (Kislak 1972; Krause et al. 2016). However, once internalised by the bacterial cell, this class of antibiotics bind to the A site within the 16S rRNA of the 30S ribosome, as can be seen in Figure 1.15 (Kotra et al. 2000; Krause et al. 2016).

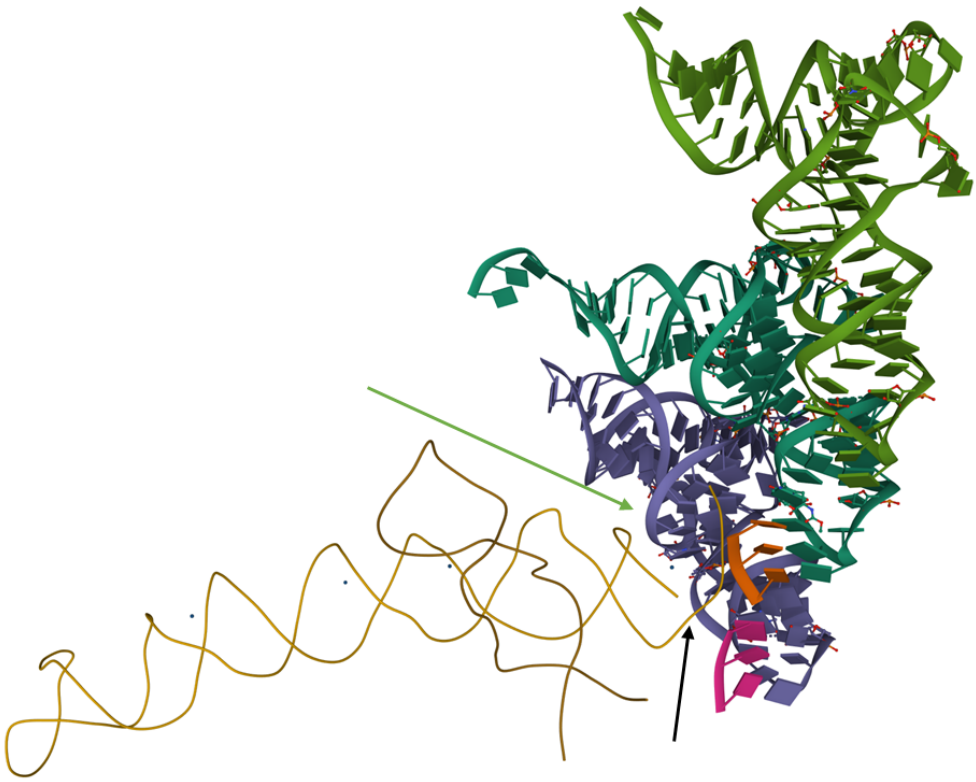


Figure 1.15 X-ray structure of 70S ribosome
This diagram shows the structure of the 70S ribosomes functional complex from the RSCB PDB identification number 486D (https://www.rcsb.org/3d-view/486D/1) (Cate et al. 1999). Visualised in *mol (Sehnal et al. 2021). The black arrow highlights the region within the 16S stem where aminoglycosides bind. The green arrow highlights the area where tRNA is bound.

Aminoglycosides bind to the penultimate 16S loop, highlighted in figure 6.2 with a black arrow, and modify the shape or the A region, (marked with a green arrow) causing a conformational change. This interrupts tRNA binding or leading to incorrect binding. This mis-synthesis, leads to an accumulation of mutant proteins, which actively encourage the uptake of aminoglycosides leading to cell death induced by the number of non-functional proteins (Kotra et al. 2000; Ramirez and Tolmasky 2010; Krause et al. 2016)

Gentamicin resistance can be induced through enzymatic modification, target site modification via an enzyme or chromosomal mutation as well as possible efflux pumps (Kotra et al. 2000; Rodvold et al. 2011; Krause et al. 2016). The primary method of aminoglycoside resistance is an enzymatic modification of the drug. These are often plasmid or integron encoded and ancestrally are produced by the same organisms producing the antimicrobial originally. This modification decreases the binding affinity thereby reducing or removing its activity. There are three primary classes of enzyme, which are aminoglycoside *N*-acetyltransferases, aminoglycoside *O*-nucleotidyltransferases, and aminoglycoside *O*-phosphotransferases. Within these classes there are hundreds of examples. The diagram Figure 1.16 shows and example of the modification of a gentamicin molecule caused by an acetyltransferase.

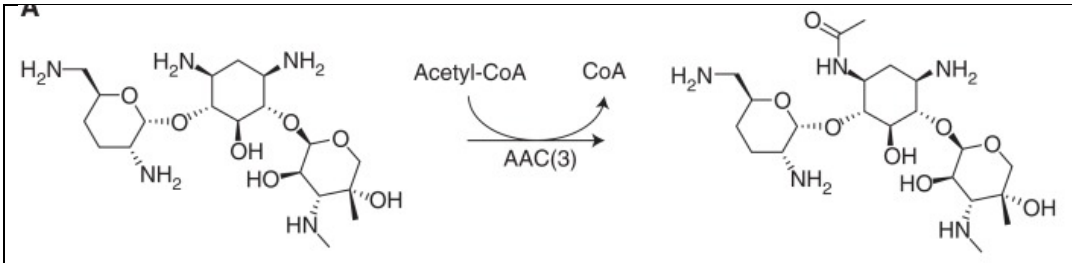


Figure 1.16 A diagram showing a gentamicin modification by an aminoglycoside acetyltransferase
Taken from: (Krause et al. 2016)

Enzymatic methylation of 16s rRNA can also be a prominent mechanism of aminoglycoside resistance. These mutations act by blocking aminoglycoside binding targets. The methylation occurs at one of two sites: either N7 or N1 position of the 30S ribosomal subunit (Krause et al. 2016), conferring high levels of resistance. Intrinsic low-level resistance to aminoglycosides can additionally be conferred through efflux pumps such as those found in some *Pseudomonas* spp. and *Burkholderia cenocepacia*, designated the MexXY efflux system.

Aminoglycosides do not concentrate within macrophages, due to their polarity and hydrophilicity (Bongers et al. 2019). In fact, inhibition using gentamicin is a quintessential laboratory technique used to specifically eliminate extracellular bacteria, to define those species that can invade eukaryotic cells. Gentamicin is primarily used topically to treat ear infections, however, it is also used empirically to treat pneumonia in hospitalised patients, administered intravenously (BNF 2021a), and is one of the most common prophylactic methods of treating neonates in intensive care unit, in combination with a beta-lactam (often colloquially referred to as treatment with "pen&gent" or "amp&gent" by clinicians). As such, it is possible that gentamicin may occasionally be used to treat undiagnosed HAI of *Legionella* pneumonia in adults that are admitted to hospitals. Although gentamicin does not enter eukaryotic cells (i.e. concentrate within a macrophage), due to its novel mode of action inhibiting protein synthesis, gentamicin was investigated regardless of its inability to penetrate macrophages.

1.13 Ampicillin

The first antimicrobial compound discovered, developed, and used clinically was penicillin, which was pioneered by Fleming, Chain and Florey. A third generation, penicillin, ampicillin remains a vital drug class after over 70 years of use (Fleming 1929). Although Paul Ehrlich and Sahachiro Hata's curing of rabbits experimentally infected with syphilis with aniline dye 606, followed by the use of "sulfa drugs" in human patients (Bosch and Rosich 2008), technically precedes this, penicillin rapidly and completely displaced the use of aniline dyes as antimicrobials in the 1940s. Ampicillin was first approved for clinical use in 1961 (Kaushik et al. 2014) and was developed to extend the functional life of penicillin-G, which had significant problems with absorption and a short half-life. Ampicillin is effective against both Gram-positive and -negative organisms, has a long half-life and can be given orally due to its acid tolerance (Kotra et al. 2000; Kaushik et al. 2014). A bactericidal compound, the mode of action for ampicillin, as well as all other penicillin's is to bind the peptidoglycan binding proteins and block peptidoglycan synthesis, leading to bacterial cell death (Yocum et al. 1979). It has been used to treat a wide variety of infections, including respiratory tract infections, since its first use (Gillespie 1965). To date ampicillin is still used to treat, UTIs, meningitis, endocarditis and streptococcal infections, and is often the first line treatment for CAP, including where the causative agent (potentially *Legionella pneumophila*) is unknown (BNF 2021a).

Ampicillin in theory has no use in the treatment of LD due to the inability of penicillin to penetrate the macrophage but there will be short periods of time immediately prior to macrophage engulfing that *Legionella* will be extracellular and available to the bactericidal effects of penicillins before *de novo* infection of new local host cells

(Shaheen et al. 2019; Valciņa et al. 2019). Modifications to ampicillin, such as being bound to nanoparticles or use of a prodrug such as pivampicillin, have also been used to overcome the poor macrophage penetration (Gehanno et al. 1996; Chanteux et al. 2003).

A defined *Legionella* spp. beta-lactamase (called LoxA) was discovered in 2002 by Avison *et al* (Avison and Simm 2002), which shows close homology to *bla-$_{oxa29}$* (originally identified in *L. gormanii*) (Franceschini et al. 2001) illustrated in clustal omega comparison in Figure 1.17 that also highlights the key areas containing amino acid changes. *loxA* is chromosomally located whereas *bla-$_{oxa29}$* is found on plasmids. *loxA* also appears to be ubiquitous across all *Legionella* species, the same is not true for *bla-$_{oxa29}$*, as plasmid content from one isolate to another varies.

```
CLUSTAL O(1.2.4) multiple sequence alignment


OXA-29          MKKLSVLLWLTLFYCGTIWAQSTCFLVQENQTVLKHEGKDCNKRFAPESTFKIALSLMGF
                60
LOX-A           MKKIIIFLCTGLLFCVSTWAQGTCFLAKENQTVLKREGNDCDQRYSPASTFKIALSLMGF
                60
                ***: ::*   *::* : ***.****.:*******:**:**::*::* ************

OXA-29          DSGILKDTLNPEWPYKKEYELYLNVWKYPHNPRTWIRDSCVWYSQVLTQQLGMTRFKNYV
                120
LOX-A           DSGILKDELHPEWPYKKEYELYLNVWKYPQNPHTWIRDSCVWYSQALTRQLGMKRFKGYV
                120
                ******* *:*******************:**:************.**:****.***.**

OXA-29          DAFHYGNQDISGDKGQNNGLTHSWLSSSLAISPSEQIQFLQKIVNKKLSVNPKAFTMTKD
                180
LOX-A           DAFHYGNQDVSGDRGQNNGLTHAWLSSSLSISPTEQIQFLQKIIYKKLPVSQKAYIMTKN
                180
                *********:***:********:******:***:*********: *** *. **: ***:

OXA-29          ILYIQELAGGWKLYGKTGNGRQLTKDKSQKLSLQHGWFIGWIEKDGRVITFTKHIADSKK
                240
LOX-A           IMYIQELPGGWKLYGKTGTGRQLTKDKSQKLPLQHGWFVGWIEKDERVITFAKHIADSKE
                240
                *:***** **********.*********** ******:****** *****:*******:

OXA-29          HVTFASFRAKNETLNQLFYLINELEK266
LOX-A           NNTFASFRAKNDALIQLFNLINELEK266
                : *********::* *** *******
```

Figure 1.17 Clustal omega generated comparison of the*bla-$_{oxa29}$* gene and the variant LoxA

A multiple sequence alignment from the bla-$_{oxa29}$ published in *Legionella gormanii* by Franceschini et al (Franceschini et al. 2001) with LOX-A from Avison's 2002 paper (Avison and Simm 2002) using Clustal Omega EMBL software (Madeira et al. 2019).

Given the use of ampicillin as a first line treatment for CAP, the advances in engineering to increase beta-lactam penetration into eukaryotic cells and the discovery of a defined *Legionella* spp. beta-lactamase (LoxA) (Avison and Simm 2002), the screening of *Legionella* spp. to ampicillin was considered to be of value.

1.14 Ciprofloxacin and Levofloxacin

The first fluoroquinolones date back to the discovery of nalidixic acid in the 1960s, which was shown to have potent antimicrobial activity against Gram-negative bacterial species (Newman et al. 1966; Zhanel et al. 1999). Nalidixic acid however was limited in its clinical use due to its rapid selection of resistant mutants, high dosage requirement, dosing frequency, photosensitivity and adverse side effects (including seizures), and most importantly poor inhibition of Gram-positive isolates (Zhanel et al. 1999).

Following nalidixic acid, the next generation represented by ciprofloxacin was a significant improvement, alleviating many of the major side-effects, increasing efficacy in Gram-negative isolates, showing effectiveness in Gram-positive isolates, and reduced (twice daily) dosing regimen. However, ciprofloxacin was still ineffective in treating Streptococcal infections and anaerobes (Newman et al. 1966; Davies et al. 1996; Zhanel et al. 1999). The only major problem with this improved fluoroquinolone was the potential for drug-drug interactions; potentially impairing renal function (Davies et al. 1996). In 1985, synthetic modification to the fluoroquinolone backbone created the 3rd generation fluoroquinolones, the prime example of which is levofloxacin, which was first used clinically in 1995 (Ball et al. 1998) The Figure 1.18, shows the basic structure of fluoroquinolones and highlights the modifications made to create levofloxacin. The main change between second and third generation is the addition of a carboxylic acid and a carbonyl group, improving binding to the gyrase complex. The alky group added in levofloxacin additionally increases its solubility.

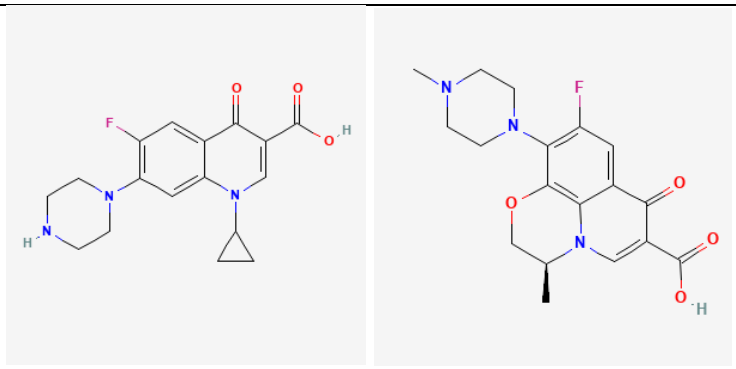


Figure 1.18 Ciprofloxacin and levofloxacin skeletal structure
https://pubchem.ncbi.nlm.nih.gov/compound/Ciprofloxacin#section=2D-Structure
https://pubchem.ncbi.nlm.nih.gov/compound/levofloxacin#section=2D-Structure

The mode of action is conserved for all fluoroquinolones. They are bactericidal antibiotics, which require passive diffusion into the target cell before acting. Once internalised in the target cell they act by binding firstly DNA gyrase, a quadrivalent protein complex consisting of two copies of GyrA and GyrB each. DNA gyrases introduce or remove helices in DNA and are an essential part of bacterial DNA replication by granting super-coiling to allow the genome to fit inside the bacterial cell, but also unwinding to allow transcription (Schmidt et al. 2010; Mustaev et al. 2014). Fluoroquinolones inhibit gyrase by binding at the joining groove between GyrA and GyrB components as can be seen in Figure 1.19.

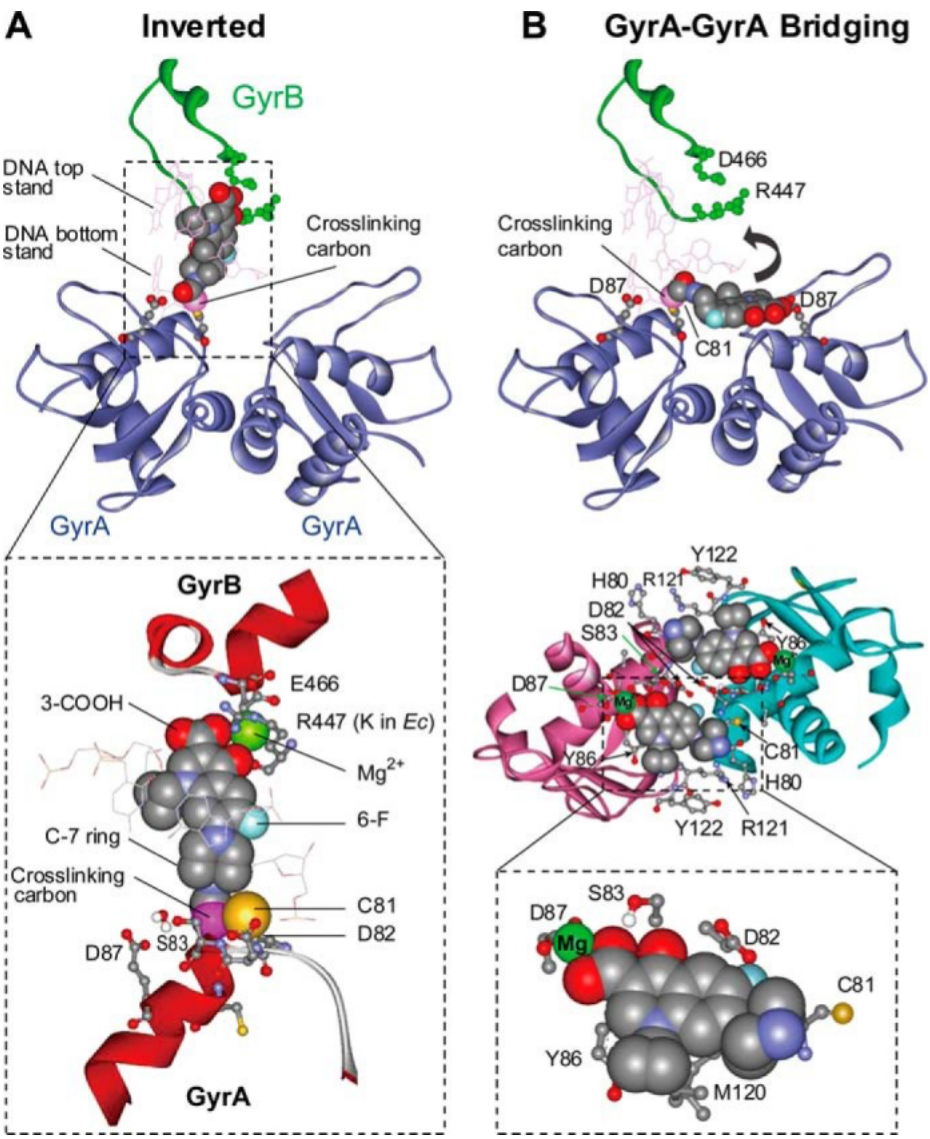


Figure 1.19 Binding between GyrA and fluoroquinolones

A, inverted configuration of the published x-ray crystal structure (PDB: 2XKK). *B*, GyrA-GyrA bridging model (PDB: 1AB4). The *top* portion shows one molecule of cross-linked ciprofloxacin in a bridging pocket. The *curved arrow* indicates the rotation needed to achieve the inverted structure shown in *A*. The *centre* portion shows two antiparallel pockets filled with fluoroquinolone. The *bottom* portion shows a detail of nearby GyrA amino acid residues; $GyrA^{87}$ and $GyrA^{81}$ are on different GyrA subunits. (Mustaev et al. 2014).

The other target for fluoroquinolones is topoisomerase IV, homologous to gyrases as it is composed of two copies of (ParC) and (ParE) each. There is strong homology of these for GyrA and GyrB, respectively, which allows for the same antibiotics to target both. Topoisomerase IV functions in the separation of dsDNA, as replication of

circular genomes results in “linked rings” that need to be cut, separated and re-ligated to allow daughter cell production. Its disruption is also bactericidal; however, it has been shown that inhibiting the GyrA/B is more potent (Zhanel et al. 1999; Fournier et al. 2000), and may underlie the greater inhibition of Gram-negative bacteria (as gyrase is the preferential target for these) relative to Gram-positive bacteria (where topoisomerase IV is the primary target of fluoroquinolones). Quinolones bind at the active site of both complexes, which is also the interactive point between DNA, the protein and the subunits. The antibiotic binds an active tyrosine; Tyr122 and Tyr120 for GyrA and ParC, respectively (Hooper and Jacoby 2016) (all numbering is as is common practice based of the *E. coli* numbering system convention).

Resistance to quinolones occurs via few well documented mechanisms as discussed. The most common, and the first discovered resistance mechanism, are non-transferable single point mutations. These are located at or near the aforementioned active tyrosine located at residues 67-106 for GyrA and 63-102 for ParC, respectively (Chen et al. 1996; Hooper and Jacoby 2016; Van Der Putten et al. 2019). These two areas are known as the QRDR (quinolone resistance determining regions) and are the most common cause of resistance in *in vitro* or *in vivo* resistant strains. The most common of these mutations is Ser83Leu in GyrA, triggering around a 24-fold increase in MIC as well as Ser80Ile in ParC; both cluster around the active tyrosine at 122 and 120, respectively. These tend to be a step wise mutation, which accumulate under exposure to sub-inhibitory concentrations of quinolones. When mutations in both GyrA and ParC are present they confer a 125-fold increase in MIC (Van Der Putten et al. 2019).

There are corresponding QRDR regions for the presence of mutations in GyrB, and its homolog ParE; however, these are much less common than those in GyrA or its

homolog ParC. They also tend to confer smaller increases in resistance. When these do occur, they tend to be focussed closer to the C-terminus of these proteins at Asp426 or Lys447 in GyrB and at Leu445 in ParE (Hooper and Jacoby 2016). Mutations which inactivate chromosomally encoded efflux pumps, such as the acrAB superfamily, have also been shown to confer resistance to ciprofloxacin, but this mutation additionally increased susceptibility to other antibiotics (Van Der Putten et al. 2019).

Mobile element acquisition of resistance has been shown through plasmid-encoded efflux pumps that trigger resistance to ciprofloxacin including *oqxAb* and *qepA*. Quinolone resistance genes known as *qnr* genes have also been reported: these reduce the binding affinity of quinolones by interacting with a DNA gyrase complex competitively interfering with quinolones ability to inhibit DNA synthesis. To date there are seven distinct genes: *qnrA-E, S and VC*. These are pentapeptide repeating proteins whose structure can be seen here: https://www.rcsb.org/3d-view/3PSS. Unusually, the aminoglycoside resistance gene family *aac(6')Ib* has also been shown to be capable of reducing the efficacy of ciprofloxacin, through expanding substrate recognition to include acetylating fluoroquinolones (Ramirez and Tolmasky 2010; Van Der Putten et al. 2019)

Ciprofloxacin has been used as a therapy since the 1980s and is still currently used to treat a wide variety of diseases, including STIs (gonorrhoea), UTIs, gastrointestinal infections, and lower respiratory tract infection, (with the exception of confirmed or expected *S. pneumoniae* diagnoses) (Davies et al. 1996).

Two fluoroquinolones were investigated here as both have roles as first line therapeutics for community and hospital acquired pneumonia (Lim et al. 2015), but also to include a 2nd and 3rd generation of this family. Prior research has shown that

they are highly effective against *Legionella* spp., partially as they both concentrate up to 5-times the external concentration within phagocytes (Wise 1991; Bongers et al. 2019). Furthermore, fluoroquinolones are of interest as there have been reports of *Legionella*, which have fluoroquinolone resistance (Bruin et al. 2014).

1.15 Tetracycline and doxycycline

The tetracycline class is a large group of antibiotics. Chlortetracycline, one of the first discovered, was isolated from *Streptomyces aureofaciens* and first marketed as a therapeutic in 1948 (Duggar 1948). Tetracycline was discovered soon after from a *Streptomyces rimosus* was FDA approved in 1954 as a more potent compound with better antimicrobial activity as well as increased solubility, as compared to chlortetracycline (Hochstein et al. 1953; Nelson and Levy 2011). Through modification of the tetracycline basic scaffold (seen in Figure 1.20) in 1967, doxycycline was first approved and remains to date one of the most used antibiotics with a broad range of function. Due to its limited side effects, it is still used for long term prophylaxis against malaria (Cunha et al. 1982; Chopra and Roberts 2001; Nelson and Levy 2011).

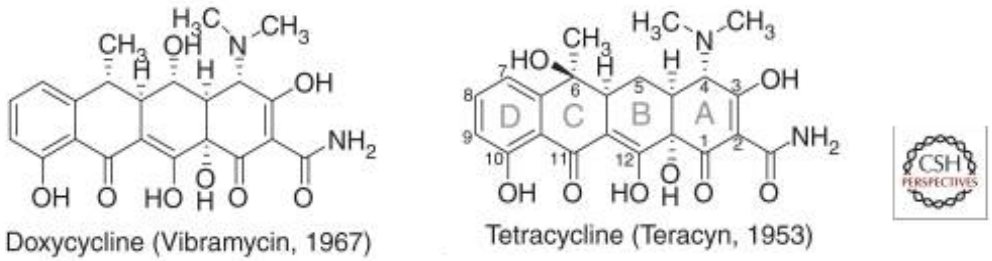


Figure 1.20 The structure of Doxycycline and tetracycline
A skeletal structure of Tetracycline and Doxycycline taken from (Grossman 2016).

As a drug class, tetracyclines have a particularly broad spectrum of function, including both Gram-positives and -negatives, spirochetes and facultative anaerobes (Slichter 2013; Grossman 2016). Tetracyclines are also able to penetrate the macrophage to some degree: enabling treatment for intracellular pathogens (Bongers et al. 2019). This work has only focussed on tetracycline and doxycycline, the former due to its global prevalence and the latter due its modern clinical usage.

The mode of action for tetracyclines is to bind the 16S rRNA component within the 30S ribosome subunit (Brodersen et al. 2000). Reversible binding prevents docking of aminoacyl-tRNA to mRNA and inhibits protein synthesis (Nelson and Levy 2011), which is a bacteriostatic function at a sufficient concentration. However, there have been reports of tetracyclines acting bactericidally (Grossman 2016) by disrupting the bacterial cytoplasmic membrane (Oliva et al. 1992). Both tetracycline and doxycycline are on the WHO list of essential medicines because of their broad spectrum, ease of oral dosing and the lack of serious side effects (WHO 2020). Within treatment of *Legionella* spp., doxycycline is commonly used to treat both hospital-acquired, and community acquired pneumoniae (Lim et al. 2009; NICE 2014; Lim et al. 2015; NICE 2019b; BNF 2021b) and while it isn't one of the suggested treatments for legionellosis, the likelihood that infected patients will receive it as an empirical therapy pre-diagnosis of legionellosis justifies the investigation below.

Resistance to tetracyclines is common, some species are innately resistant, and others use the plethora of acquired resistance genes, discussed below. The methods and genes conferring resistance are shown in the figure taken from (Markley and Wencewicz 2018).

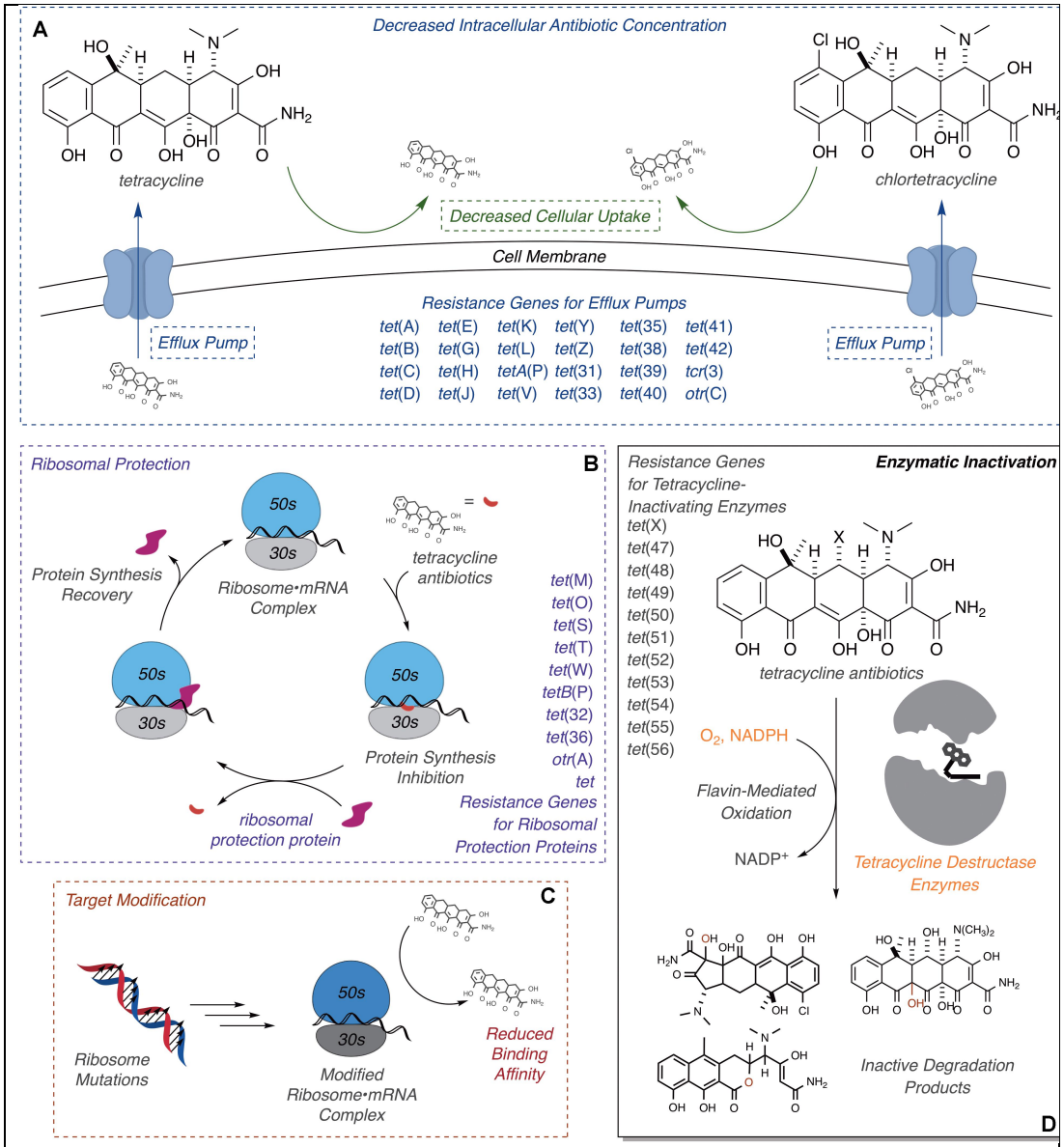


Figure 1.21 Tetracycline resistance mechanisms

(A) Efflux, exclusion, (B) ribosome protection, (C) ribosome modification, and (D) enzymatic inactivation. With associated ARGs with mode of resistance provided. Taken from: (Markley and Wencewicz 2018).

Firstly, resistance-mediating mutations within the16S rRNA are most commonly found in bacteria with fewer copies of rRNA (*Legionella* spp. has three). These mutations often abrogate tetracycline's ability to bind and mutations generally cluster around the four tetracycline binding sites Tet-1-4. However, other mutations can also potentially confer resistance as they may induce a conformational change in the tertiary structure, preventing tetracyclines from binding through three-dimensional

changes inducing inhibition of molecular access to the binding site. Below is a table and figure highlighting some of the key mutations presented by Grossman in 2016.

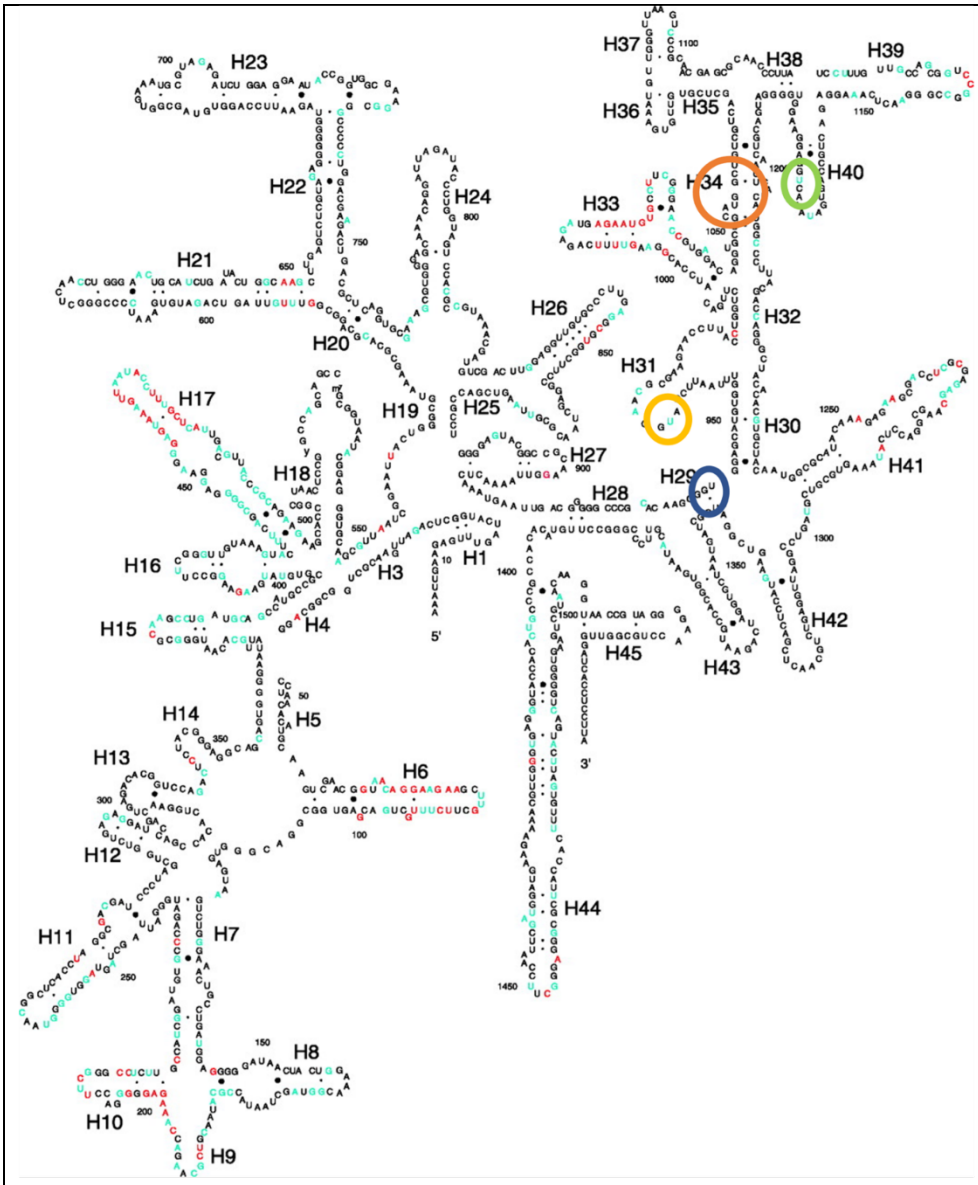


Figure 1.22 16s RNA structure highlighting key areas of mutation conferring tetracycline resistance

This skeletal structure of the 16S rRNA numbered (using *E. coli* numbering scheme) every 50 nt. The circles represent key areas of resistance for tetracyclines. Yellow represents the Tet-1 primary binding point and blue, the Tet-4 binding point. Mutations in the orange, and green circles causes conformational changes, rather than direct interference with tetracycline binding. Information taken from (Grossman 2016) figure modified from (Case et al. 2007).

Table 7 Highlighting resistances to tetracyclines induced by 16S RNA mutations

Bacteria	Mutation	Location
Helicobacter pylori	AGA 965-967 TTC G942	H31 loop Tet-2
P. acnes. M bovis	G1058C	H34
M. bovis	A965T A967T/C U1199C	H31
S. pneumoniae	C1045T T1062G/A	H34

(Grossman 2016)

The next method of resistance is acquisition of tetracycline-specific ribosomal protection proteins (RPPs). They are enzymes which can dislodge tetracycline from binding to the ribosome allowing the translation to continue in the presence of tetracycline. There is some literature, which suggests that they also induce a conformational change to the 30S ribosomal subunit through phosphorylation that additionally prevents tetracycline rebinding (Connell et al. 2003). The two major genes were named *tet(M)* and *tet(O)* (as seen in Figure 1.21) first described in *Campylobacter jejuni* and *Streptococcus* spp., respectively. These genes confer a high degree of tetracycline resistance, on top of which being commonly encoded on plasmids; they are widely disseminated in Gram-positive and -negative organisms through horizontal gene transfer (Connell et al. 2003; Li et al. 2013),

Efflux pumps are ubiquitous and there are over 30 reported distinct tetracycline specific efflux pumps reported, and they are curated by Roberts (http://faculty.washington.edu/marilynr/) (Roberts 2022). Genes *tet(A)* and *tet(B)* are the most commonly reported efflux pumps in Gram-negative isolates and confer medium to high levels of resistance against many different tetracyclines, often with the exception of tigecycline (Grossman 2016).

The last method of tetracycline resistance is enzymatic inactivation. These are antibiotic destructases. These enzymes function to physically cleave the antibiotic,

rather than modify through addition of acyl-, nucleotide, or phosphate group, as their mechanism of resistance (Wright 2005; Markley and Wencewicz 2018). Most antibiotics have a destructase, which renders them ineffective (Markley and Wencewicz 2018). This resistance method is the only one, which not only reduces intracellular concentrations of the antibiotic, but also reduces the extracellular concentrations as well (these enzymes can also be secreted extracellularly) (Markley and Wencewicz 2018). The best-known examples of antibiotic destructases are beta-lactamases, which destroy the lactam ring within penicillin. These destructases are often carried on plasmids and are easily transferable (Davies 1996). The gene *tetX* is the first and best known of the tetracycline destructases. First reported in 1989, it was isolated from *Bacteroides fragilis* (Speer and Salyers 1989). *tetX* and all other tetracycline destructases are single component flavoprotein hydroxylases which degrade tetracyclines backbone using NADPH as an electron donor to oxidise tetracycline, as can be seen in Figure 1.23.

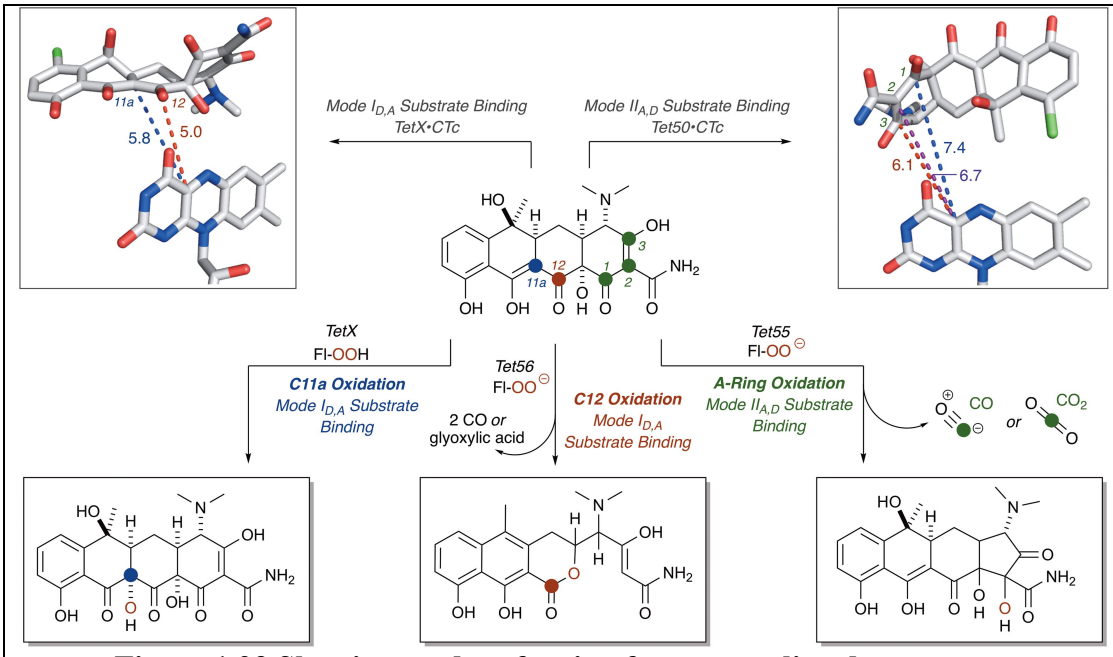


Figure 1.23 Showing modes of action for tetracycline destructases

Figure showing the site of tetracycline oxidation with variations on the Tet gene taken from: (Markley and Wencewicz 2018)

Soil bacteria, both culturable and unculturable, have long been associated with both antibiotics and resistance to those antibiotics; as such discovering novel antibiotic resistance mechanisms is not common. However, for tetracycline, for which the intrinsic resistance remains high, the prevalence of known and or close homologs of resistance genes are rare. This has been postulated to be because the existing proteins are highly active enzymes with wide substrate recognition (which can target multiple compounds), or because the undiscovered modes of activity are so different, they are currently unrecognisable as resistance determinants (Davies 1994; Nesme et al. 2014). Some success has been achieved in screening of soil bacteria, finding novel tetracycline destructases such as Forsberg *et al,* who demonstrated nine new tetracycline destructases with a maximum homology to Tet(X) of 70% from soil bacteria. This group also discovered a tenth destructase in the human pathogen *Legionella longbeachae* by genomic screening. (Forsberg et al. 2015). The *Legionella longbeachae tetX* was named *tet(56)* and is illustrated in the Figure 1.23 as the middle tetracycline destructase, showing its mode of action in oxidising and breaking down the compound. The PyMol crystal structure in the Figure 1.24 shows *tet(56)* extracted from a *Legionella longbeachae* strain and expressed in an *E. coli* surrogate. It was shown to significantly increase tetracycline resistance in this transfected *E. coli* while being used to generate large quantities of purified protein for x-ray crystallography studies (Park et al. 2017; Markley and Wencewicz 2018). *tet(56)* has been found ubiquitously in all *Legionella longbeachae,* and no close homologs have been found in any other *Legionella* species.

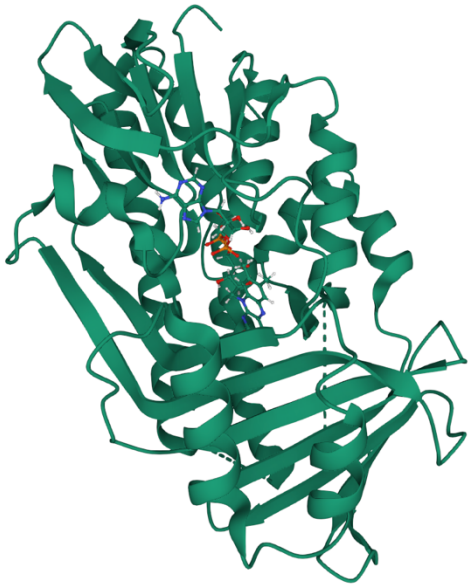


Figure 1.24 Crystal structure of *tet(56)*
A schematic of the structure of Tet(56) as found by (Park et al. 2017), schematic made in PyMol: (Sehnal et al. 2021) https://www.rcsb.org/3d-view/5TUM

1.16 Chloramphenicol

First introduced in 1949 as a broad-spectrum antibiotic (Feder et al. 1981), this drug's importance, like colistin, has waxed and waned, but it is gaining relevance again due to resistance against other compounds and classes of antibiotics. It is primarily used to treat minor eye infections as well as otitis externa, both applied as drops, which have few and infrequent side effects (NICE BNF 2021). Chloramphenicol can also be prescribed orally or intravenously, often to treat severe meningitis and or septicaemia most often caused by *Haemophilus influenzae* and typhoid fever (S*almonella typhi*). However, chloramphenicol has significant albeit rare side effects including aplastic anaemia and can cause grey baby syndrome in neonates (Scholar 2007), and as such is rarely prescribed. Its mode of action is unique, competitively (and reversibly) binding aminoacyl-tRNA in the 50S subunit of the ribosome, which changes the ribosome inhibiting transpeptidation (Drainas et al. 1987). Resistance to chloramphenicol is most often enzymatically driven by a chloramphenicol acetyltransferase or phosphotransferases. These are widely disseminated and often found on plasmids (Fernández et al. 2012; van Bambeke et al. 2017). Resistance is also seen by target modification, reducing the membrane permeability and efflux pumps, much as I have shown in sections above (Fernández et al. 2012). Chloramphenicol was evaluated due to its novel mode of action, its good cellular penetration, unlike ampicillin or gentamicin (Davey et al. 2015), and the general dearth of information on its efficacy against *Legionella* spp.

1.17 Azithromycin

Macrolides belong to a large family of drugs called macrocyclic antibiotics that can be divided into four groups: macrolactams or anamycins (including rifampicin), polyene macrolides (antifungal antibiotics including nystatin that have no effect on bacteria), macrolide-like compounds and macrolide antibiotics. Macrolide antibiotics share a common core structure, characterized by large lactonic cycle with 12, 14, 15 or 16 carbons, to which sugar- and/or aminosugar-moieties are bound. Colloquially the term macrolide is usually used to refer to antibiotics composed of 14- or 15-membered lactones (i.e., erythromycin, clarithromycin, or azithromycin) a very occasionally 16-membered lactones (i.e., josamycin). All of these are decorated with sugars via glycosidic bonds (Roberts et al. 1999). The first macrolide, erythromycin, was clinically introduced in the early 1950s with resistance reported within a year (Weisblum 1995). Azithromycin is a modified form of erythromycin first used in 1981, which has improved physiological uptake as well as a reduced side-effects compared to erythromycin. All macrolides, azithromycin included, are protein synthesis inhibitors working through the binding of the 50S subunit of the bacterial ribosome, stopping the translation of mRNA, by blocking the exit tunnel of the ribosome. Macrolides are bacteriostatic (stalling replication while present at a sufficient concentration; reversible) rather than bactericidal (mediating bacterial death; irreversible) (Foulds et al. 1990). Azithromycin has been found to concentrate within phagocytes and at concentrations ≥50 times the levels reached in plasma have been reported and as such the pharmacokinetic-pharmacodynamic (PK:PD) probability of target attainment will invariably be higher (Stamler et al. 1994). Azithromycin is on the WHO list of essential medicines and is widely used due to its

potency and broad spectrum of activity (WHO 2020). Its efficacy in *Legionella spp.* treatment is enhanced by its accumulation within a macrophage. Globally it is used as a treatment for CAP and SCAP (Severe Community Acquired Pneumonia) (Campèse et al. 2015) and in the UK the NICE guidelines recommend its use in SCAP or as a second line treatment option for non-responsive CAP (BNF 2019).

Azithromycin resistance is most often caused by ribosomal modification or methylation preventing binding, efflux pumps or drug inactivation. Ribosomal methylation triggered by the one of 40 published *erm* (erythromycin ribosome methylase) genes widely reported (Farrow et al. 2000), act by adding one or two methyl groups to an adenosine in the 23S rRNA of the 50S ribosomal subunit (Leclercq 2002). The adenosine located at A2058 is a key residue in the 23S subunit and the usual key target of binding for macrolide and lincosamides; therefore, its methylation leads to a direct steric hindrance for antibiotic binding and often confers a broad range of resistance to macrolides and lincosamides. The key genes within this subset are *erm(A), erm(B), erm(C),* and *erm(F)* (Leclercq 2002). These genes can either work continuously (whereby they are constantly expressed) which has a higher fitness cost, but also confers higher degrees of resistance, or are induced after induction triggered by low levels of macrolides. These have a lower long term fitness cost, but only confer resistance to macrolides after the inducing attenuator (Farrow et al. 2000). Erm genes in *Legionella* species have not been reported to date.

The last mechanism of macrolide resistance relates to physical modification of the drug, rather than the host. Formed mainly by esterases and phosphotranspherases (Leclercq 2002), these enzymes specifically target defined substrates sometimes recognising only some members of an antibiotic species. For example, number of *lnu* genes have been described in different bacteria and these include *lnu*(A) (Brisson-Noël

and Courvalin 1986), *lnu*(B) (Lüthje and Schwarz 2007), *lnu*(C) (Achard et al. 2005), *lnu*(D) (Petinaki et al. 2008), *lnu*(E) (Zhao et al. 2014) and *lnu*(F) (Heir et al. 2004); while these *lnu* genes confer resistance to lincomycin and pirlimycin, the increase in MIC against clindamycin are often below the resistance breakpoints (Leclercq et al. 1987; Zhao et al. 2014)

Another common mechanism of impeding macrolide binding to the 23S rRNA subunit is via induction of site-specific mutations and are a common cause of macrolide resistance. Mutation of adenosines to guanosine often at positions A2058 or A2059 region of the V domain in rRNA are the most common, but as can be seen in Figure 1.25 mutations can occur outside these regions (Vester and Douthwaite 2001; Leclercq 2002) These mutations tend to be consistent across species and traditionally referenced against E coli 23S rRNA gene numbering, which is why the rRNA represented below is from *E. coli.* Interestingly, these mutations can often also mediate resistance to lincosamides, pleuromutilins and chloramphenicols.

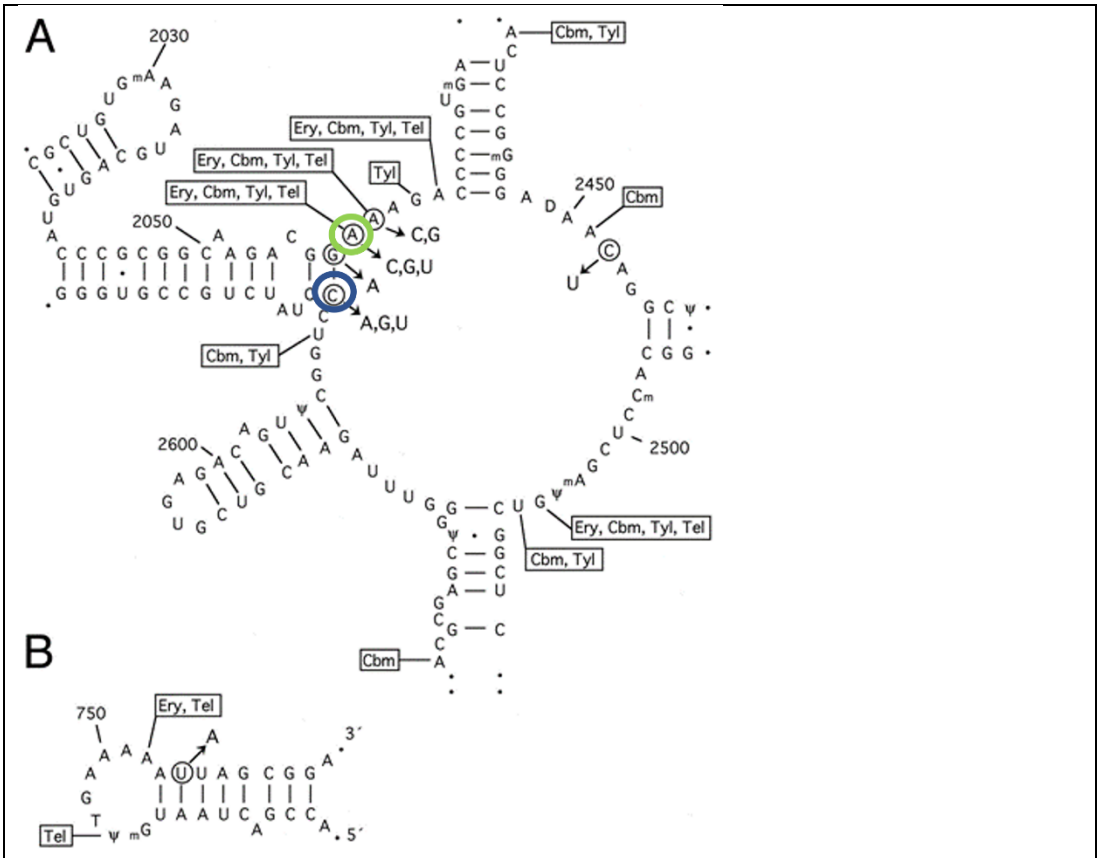


Figure 1.25 Showing the Secondary-structure models domain V of 23S rRNA (A) and hairpin 35 in domain II (B) with nucleotides at which macrolide drugs interact highlighted.

Azithromycin Azm, Carbomycin Cbm, Clarithromycin Clr, Erythromycin Ery, Telithromycin Tel, Tylosin Tyl. Modified from: (Vester and Douthwaite 2001)
Green highlights 2058 and Blue 2611 the two key mutation points for azithromycin resistance in *Legionella pneumophila* as shown by (Descours et al. 2017). Numbering as per *E. Coli* convention.

The ribosomal RNAs exist as consecutive genes: 16S, 23S and 5S, as a cluster in the genome and this is referred to as the *rrn* operon. Some bacteria have a single operon (*Mycoplasma genitalium*) while some bacteria have five or six operons; generally, the length of the genome relates to the number of operons encoded. For *Legionella* spp. genomes routinely contains three *rrn* operons. Mutations are not necessarily needed in all operons to confer resistance, but as the number of operons with resistance-mediating mutations increases- the degree of resistance also increases (Descours et al.

2017). Furthermore, *rrn* mutation as a mechanism of resistance is usually only effective for genomes that have three or fewer *rrn* operons.

In 2017 a study published by Descours *et al* showed that through serially passaging of *Legionella pneumophila* in increasing concentrations of macrolides it was possible to induce resistance MIC up to 4096-fold above the starting MIC concentration. Repeated sequencing of these isolates as they were passaged revealed three key points of mutation within the three *rrn* operons, in the L4 protein and/or the L22 protein. Only two mutations in the *rrn* operons were found: C2611T, A2058G and one or the other of these occurred on every high MIC strain. The number of mutations in the three *rrn* operons correlated with an increase in MIC. The highest number of passages carried resulted in mutations in all three *rrn* operons. Within the L4 protein mutations were conserved across two points and were found at the: T65K and G66R/A/C as well as a deletion of $_{63}$KG$_{64}$. T65K was ubiquitous across each passaged isolate with the exception of the sole isolate containing the $_{63}$KG$_{64}$ (Descours et al. 2017). This investigation into these three areas were critical in determining the reasons for high MIC in isolates that did not contain other known resistance genes.

Efflux pumps are transport proteins which move a substrate across the membrane from the inside of the cell to the outside and they are ubiquitous among bacteria. An efflux pump may only work for one substrate or they may have a wide range of dissimilar substrates (Webber and Piddock 2003). Efflux pumps are mostly chromosomally encoded and all bacteria (bar mollicutes- as they lack a cell wall) contain multiple examples, there are also examples of plasmid-encoded efflux pumps (such as *msr(A)* in *S. epidermidis*) (Ross et al. 1990). In Gram-negative species the chromosomally encoded pumps target hydrophobic compounds which can include macrolides. These

pumps, in general, are ancestrally linked and around 5% of chromosomal genes are involved in encoding of efflux pumps (Saier and Paulsen 2001). These intrinsic efflux pumps are controlled by the host through transcriptional regulators that increase and decrease expression; when over-expressed these efflux pumps result in multi-drug resistance (Leclercq 2002).

An efflux pump encoded by the gene *lpeAB* confers reduced susceptibility to azithromycin in *Legionella pneumophila*, as demonstrated by Massip *et al* in 2017. They demonstrated that following transfer of the *lpeAB* gene to *L. pneumophila* strains, the resultant bacteria had up to 16-fold higher MIC for azithromycin (reproduced in Figure 1.26). This publication also demonstrated that sub-inhibitory concentrations of macrolides promoted resistance-mediating mutations in the upstream promotor regions which increased resistance (Massip et al. 2017). This may account for variations in the degree of resistance mediated by strains containing the *lpeAB* gene, which can vary from minor increases to more marked increases for macrolide MICs relative to non-*lpeAB* carrying strains.

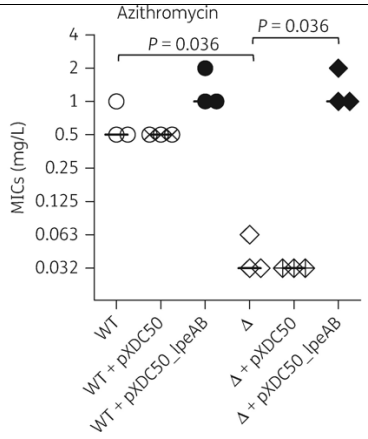


Figure 1.26 Effect of *lpeAB* depletion and complementation in *L. pneumophila* Paris

This figure shows the effect of *lpeAB* depletion and complementation in *L. pneumophila* Paris on MIC to azithromycin. Black lines represent the median of three experiments.
On the left the wildtype LP Paris WT, WT+pXDC50 and WT+pXDC50_*lpeAB*,
On the right the WT strain (Paris) which has had the inherent *lpeAB* gene knocked out creating Δ showing the MIC of Δ, Δ+pXDC50 and Δ+pXDC50_*lpeAB*. Edited from Massip *et al* 2017 (Massip et al. 2017).

In Figure 1.26 knock-out and reintroduction of the *lpeAB* gene suggests that *lpeAB* had a significant effect on the MICs of this strain of *Legionella* to azithromycin. As such the presence/absence of *lpeAB* would be a resistance mechanism of interest to azithromycin. This gene is a homolog of the multidrug resistance causing efflux pump gene *acrAB* found in *E. coli* (Yu et al. 2003; Massip et al. 2017).

1.18 Rifampicin

A polyketide ansamycin, rifampicin is primarily used to treat a *Mycobacterium tuberculosis* (TB) infection. Its mode of action is bactericidal through the inhibition of DNA-dependant RNA synthesis mediated via binding of the subunit B of the RNA polymerase. Rifampicin administered *in vivo* reaches a concentration between 1-2.3µg/mL in the lungs (Kiss et al. 1976). Rifampicin has been shown to penetrate macrophages up to 20-fold higher than found in plasma (Ziglam et al. 2002; Rodvold et al. 2011).

A review published in 2011 by Varner *et al* assessed the combinational therapeutic uses of rifampicin (Varner et al. 2011). Its bactericidal properties are of particular importance when dealing with immunocompromised patients who are already at a higher risk of morbidities and mortality from LD, because of their reduced ability to clear infection, whilst growth of *Legionella* spp. are only stalled by bacteriostatic macrolide antibiotics like azithromycin until the localised concentrations diminishes.

Rifampicin has never been considered for use as a monotherapy in LD for three reasons:

1. Resistance to rifampicin develops quickly (Nielsen et al. 2000)
2. Rifampicin is nephrotoxic, hepatotoxic, can be associated with idiopathic thrombocytopenia
3. Rifampicin is kept by the WHO in reserve where possible for use in the treatment of drug resistant TB.

A study by Baltch *et al* demonstrated high degrees of antibacterial synergy when combinations of rifampicin and levofloxacin were used at a concentration of $1/10^{th}$ of the mono-therapeutic MIC for both antibiotics, to a lesser degree rifampicin and

erythromycin also showed these synergistic antimicrobial properties (Baltch et al. 1995).

Table 8 *In Vitro* Data on Rifampicin Bioactivity Against *Legionella pneumophila*

Showing trials investigating rifampicin combination therapies, highlighting the overall lack of efficacy and potentially increased severe side effect associated with combination therapies. Modified from(Varner et al. 2011)

Reference	Study type	Treatment (=number of patients)	Results
Dournon 1990	Review Severe LP cases Endpoint Mortality	Erythromycin (20) Erythromycin/Rifampicin (20) Pelloxacin/erythromycin, (20) Pelloxacin (7) (subset of pelloxecirv erythromycin group)	Mortality 50% erythromycin alone. 40% combination. 28.6% pefloxacin monotherapy
Hubbard 1993	Review Severe LP cases Endpoint Mortality	Erythromycin, (11) Erythromycin/rifampicin, (15) No antibiotics, (4)	Mortality 27% (3/11) with erythromycin alone vs 33% (5/15) with combination. jaundice more likely with combination (p=0.028)
Howden 2003	Retrospective observational Confirmed LP cases Endpoint Mortality	Combination, (79) (19% with rifampicin) Monotherapy, (25)	Overall mortality 5.7% (6/104), none of which received combination therapy with rifampicin Treatments not associated with end points
Mykletiuk 2005	Observational review Confirmed LP CAP cases Endpoint Mortality	Macrolide, (32) Macrolide/Rifampicin, (48) Fluoroquinolone, (40)	Overall mortality 5%; no significant difference between groups Significant improvement in clinical endpoints with fluoroquinolone
Blszquez Garrido 2005	Observational prospective, non-randomised Confirmed LP CAP cases Endpoint Mortality	Levofloxacin, (45) Levofloxacin/ rifampicin, (45)	1 death in levofloxacin arm Fewer complications and improved clinical outcomes with levofloxacin alone
Grau 2006	Observational cohort Confirmed LP cases Endpoint Mortality	Clarithromycin, (11) Clarithromycin/ Rifampicin, (21)	All patients cured Length of stay increased in combination arm; 8.4 vs 12.4 days (p=0.035)

In the six *in vivo* trials, reported in Table 8 reviewed in Varner *et al,* minor adverse events were reported including a significant (p=0.028) increase in jaundice as well as a significant (p=0.035) increase in length of stay for patients treated with rifampicin combinations (Varner et al. 2011). This compares to an overall benefit of fewer complications and better patient outcomes for levofloxacin monotherapy.

Rifampicin is recommended for use in the treatment of Legionnaires' disease by the BNF (British National Formulary) at a dose of 0.6-1.2g/day (BNF 2019) to be used in combination with another antibiotic most often ciprofloxacin or azithromycin. Its use is not recommended by the British thoracic society nor stated in the NICE guidelines for the treatment of CAP or SCAP, primarily due to its ascribed nephrotoxicity. In France it is occasionally recommended alongside macrolides or fluoroquinolones as a combination therapy for immunosuppressed patients mainly as it increases the bactericidal effects of both aforementioned drug classes (Campèse et al. 2015).

2 Aims and hypothesis

Antibiotic resistance is a global health concern and due to the current culture methods for *Legionella* it is not possible to routinely screen for resistance. I hypothesised that this meant that *Legionella* resistance would be under reported and under investigated.

First aim: To devise a charcoal free solid agar, which was suitable for high throughput screening to investigate the antimicrobial sensitivity of *Legionella*.

Second aim: Once the novel media (LASARUS) had been invented, to compare results to the gold standard of serial broth microdilution (BMD), and to determine the degree of inaccuracies incurred through us of BCYE-α in agar or disk diffusion.

Third aim: To screen the entire UKHSA archive (~2,100 *Legionella* isolates) to determine the normal distribution of MIC and identify outliers and elucidate their modes of resistance.

Fourth aim: To publish an international position paper setting out recommendations for the standardization of antimicrobial susceptibility testing methods, guidelines, and reference strains to facilitate an improved era of antibiotic resistance determination.

3 Materials and Methods

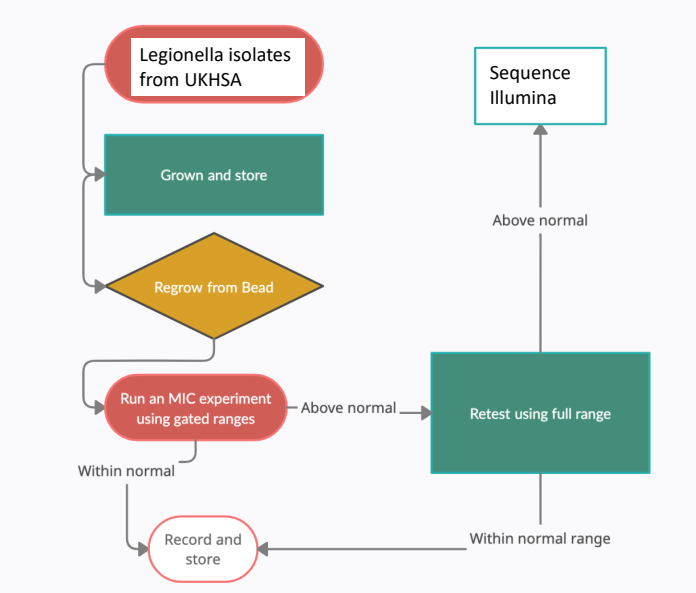


Figure 3.1 The AST isolate workflow
Workflow for the processing of samples from initial arrival in Cardiff to storing, running MIC experiments and then preparation for sequencing to investigate underlying causes of resistance made using Creately).

3.1 Media preparation

3.1.1 BCYE-α

Making BCYE-α was a two-step process. 12.5g of BCYE-α base (Sigma, UK) was resuspended in in 450mL of ddH_2O and autoclaved. Once autoclaved it was cooled in a water bath to 50ºC, mixed with *Legionella* supplement (Sigma, UK) (also at 50ºC) and 0.2g of filter sterilised L-Cysteine and poured into petri dishes.

Table 9 Showing the reagents needed to make 500mL of BCYE-α

Reagent g/L	BCYE-α g	Autoclaving required
Yeast extract	5	Yes
ACES buffer	5	No
KOH 1N	1.4	No
Agar	6	Yes
Purified water	To 500mL	Yes
Ferric pyrophosphate	0.125	No
L-Cysteine	0.2	No
Alpha Ketoglutarate	0.5	No
Activated charcoal	1	Yes

For some experiments, selective agents were also added such as amphotericin B, vancomycin and colistin. However, there is evidence that they reduce *Legionella* growth (Ditommaso et al. 2021), as such they were not included in AST plates.

3.1.2 LASARUS

Making LASARUS was a two-step process.

First 5g of select agar (Sigma, UK) was resuspended in 200mL of ddH_2O and autoclaved. Once autoclaved it was cooled in a water bath to 50°C.

Secondly a mixture of 5g of vitamin enriched yeast extract (Sigma, UK), one pre warmed to 50°C vial (50mL) of *Legionella* growth supplement (Sigma, UK), 50mL of heat inactivated sheep serum was mixed with 200mL of ddH_2O. To this was added 0.2g of L-cysteine resuspended in 5mL of ddH_2O and filter sterilised using a 0.22µm filter. This mixture was then filter sterilised using a 500mL filter steriliser (Corning, USA) attached to a powerful suction pump (Sigma, UK). This was then pre-heated to 50°C.

Parts A and B were then mixed and poured into the required agar plates, 20mL per 90mm round petri dishes and 35mL per 100mm square plates.

For the improved formulation of LASARUS pH adjustments were carried out on the liquid component of the media pre-sterilising, using a pH probe (Hanna, pH 210 Microporcessor pH Meter). The pH was reduced from 7.2 to 6.9 using H_2SO_4 if the pH needed increasing NaOH was used. The liquid phase of LASARUS was used rather than the final mix as I did not own an agar surface pH reader, five plates shipped to UKHSA were read using a surface agar reader and the pH was the same as when tested by liquid probe. Compound E was then added to ensure a final concentration of 471µg/mL.

3.2 Legionella strains

All *Legionella* strains including clinical isolates, environmental isolates, typed serogroup strains, a panel of NCTC (National Collection of Type Cultures (UK)) strains and panel of all *Legionella* species were provided by Dr Victoria Chalker, Head, Respiratory and Vaccine Preventable Bacterial Unit, UKHSA. These were part of the archive collection (PHE) now UKSHA (UK Health Security Agency). They were shipped under UN3373 conditions on dry ice, grown on BCYE-α agar plates and beaded using cryogenic vials (Technical Service Consultants Ltd (TSC), UK) and stored at –80ºC.

3.3 Culturing Legionella species

3.3.1 Charcoal swab

A charcoal swab containing the transported *Legionella* was streaked over one quarter of a BCYE-α plate with a plastic 1µL loop used to pick a single colony (see Figure 3.2 below). Plates were then incubated in a humidified atmosphere at 37ºC for five days, after which a single *Legionella* colony could be picked and either frozen on a TSC bead or utilised in experiments.

3.3.2 Cryovial

From a frozen cryovials, a single bead was streaked over one quarter of a BCYE-α plate, then using a plastic 1µL loop the inoculum was streaked to obtain single colonies (see Figure 3.2). The plate was then incubated in a humidified atmosphere at 37ºC for five days. After which a single *Legionella* colony could be picked and either frozen on a TSC cryo-bead or used for MIC determination, comparative growth experiments, or scaling up for sequencing.

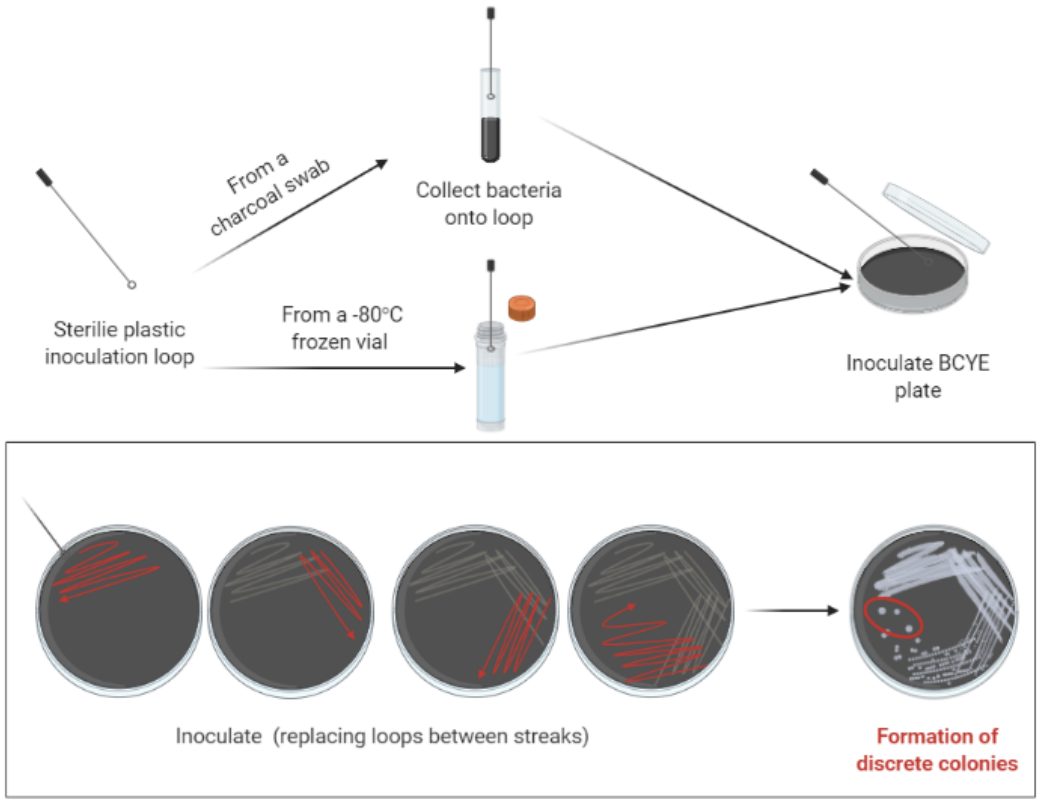


Figure 3.2 Inoculating *Legionella* onto a BCYE-α agar plate
Inoculating *Legionella* using a sterile plastic loop from a charcoal swab or a frozen bead to streak onto a BCYE-α agar plate with the aim of growing single colonies of *Legionella*.

3.4 MIC General

MIC experiments were a fundamental part of this research project and were broken down below into three main parts, 3.4.1, 3.4.2 and 3.4.3. With an additional section on the modifications made for broth-based MIC experiments.

3.4.1 Bacterial suspensions

Single colonies were picked from a BCYE-α plates incubated as described in 3.3 were resuspended in 3mL of sterile H_2O (Thermo Fisher Scientific, USA) at a concentration equivalent to 0.5 McFarland (expected to produce an inoculum of approximately

$1.5x10^8$ CFU/mL). A 200µL volume of 1:10 dilution of the 0.5 McFarland solution was made for each isolate suspension using ddH_2O and placed in a 96-well cell culture plate (Greiner Bio-One, Germany).

3.4.2 Antibiotics

Antimicrobials used included: rifampicin, levofloxacin hydrochloride, doxycycline hydrochloride (Sigma–Aldrich, Poole, UK) and azithromycin (Aspire Pharma, UK) see Table 12. All working stocks were made fresh at a concentration of 2560µg/mL in 10mL in ddH_2O.

For rifampicin, 25.6mg was initially dissolved in 1mL of DMSO, which when dissolved was then added to 9mL of ddH_2O.

Doxycycline, levofloxacin, and azithromycin were dissolved initially at 100mg/mL, as per the manufacturers' instructions, before dilution in H_2O to working stocks. All other antibiotics were made direct to 2560µg/mL by re-suspending 25.6mg of each antibiotic with 10mL of ddH_2O. All antibiotics were stored at 4°C apart from doxycycline, levofloxacin, and azithromycin, which were stored at -20°C once resuspended in H_2O. Gentamicin was purchased as a pre-prepared liquid at 10,000µg/mL and was diluted to 2560µg/mL.

Once reconstituted to 2560µg/mL additional stocks were made at 80, 2.5 and 0.078125µg/mL (Table 10) to both minimise pipetting error and ensure volume range was acceptable.

Following this preparation step, the continuous range of antibiotics for the agar dilutions were prepared as detailed in Table 9. Pre-labelled 50mL falcon tubes were utilised to give the required final antibiotic concentration ranging from 256-

0.001µg/mL in a volume of 40mL. LASARUS or BCYE-α agar at 50ºC were then added to these falcon tubes to make up to the final 40mL volume, gently mixed (to minimise bubble formation) and then poured into a single 100mm square plate (Sarstedt, Germany) (i.e. one antibiotic, one concentration, one plate).

Table 10 Antibiotic stock concentration

Stock µg/mL	Stock use µL	End volume µL	End concentration µg/mL
2560	10000	10000	2560
2560	500	16000	80
80	500	16000	2.5
2.5	500	16000	0.078125

Table 11 Antibiotic stocks for agar dilution protocol

Stock µg/mL	Stock use µL	End volume µL	End concentration µg/mL
2560	4000	40000	256
2560	2000	40000	128
2560	1000	40000	64
2560	500	40000	32
2560	250	40000	16
2560	125	40000	8
80	2000	40000	4
80	1000	40000	2
80	500	40000	1
80	250	40000	0.5
80	125	40000	0.25
2.5	2000	40000	0.125
2.5	1024	40000	0.064
2.5	512	40000	0.032
2.5	256	40000	0.016
2.5	128	40000	0.008
0.078125	2048	40000	0.004
0.078125	1024	40000	0.002
0.078125	512	40000	0.001
0.078125	256	40000	0.0005
0.078125	128	40000	0.00025
0.078125	64	40000	0.000125

Table 12 Antibiotics used for MICs

Antibiotic class tested	Antibiotic	Source of antibiotics
Macrolide	Azithromycin	Aspire Pharma, UK
Aminoglycoside	Gentamicin	Sigma–Aldrich, Poole, UK
Phenicol	Chloramphenicol	Sigma–Aldrich, Poole, UK
Fluoroquinolones	Levofloxacin	Sigma–Aldrich, Poole, UK
Fluoroquinolones	Ciprofloxacin	Thermo Fisher Scientific, UK
Tetracyclines	Tetracycline	Sigma–Aldrich, Poole, UK
Tetracyclines	Doxycycline	Sigma–Aldrich, Poole, UK
Ansamycin	Rifampicin	Sigma–Aldrich, Poole, UK
Penicillin	Ampicillin	Sigma–Aldrich, Poole, UK

3.4.3 Inoculation and reading results

All media containing the test range of antimicrobials (and an antimicrobial-free growth control) were inoculated from a standardized inoculation plate using a

multipoint inoculator (MAST URIVRDOT, Mast Group, UK). Multipoint inoculator pins were sterilized in 80% ethanol for 10mins prior to inoculation. Once inoculated, plates were left to dry for between 15mins and 30mins to ensure the spots of inoculation had dried then inverted and incubated as above and read after five days.

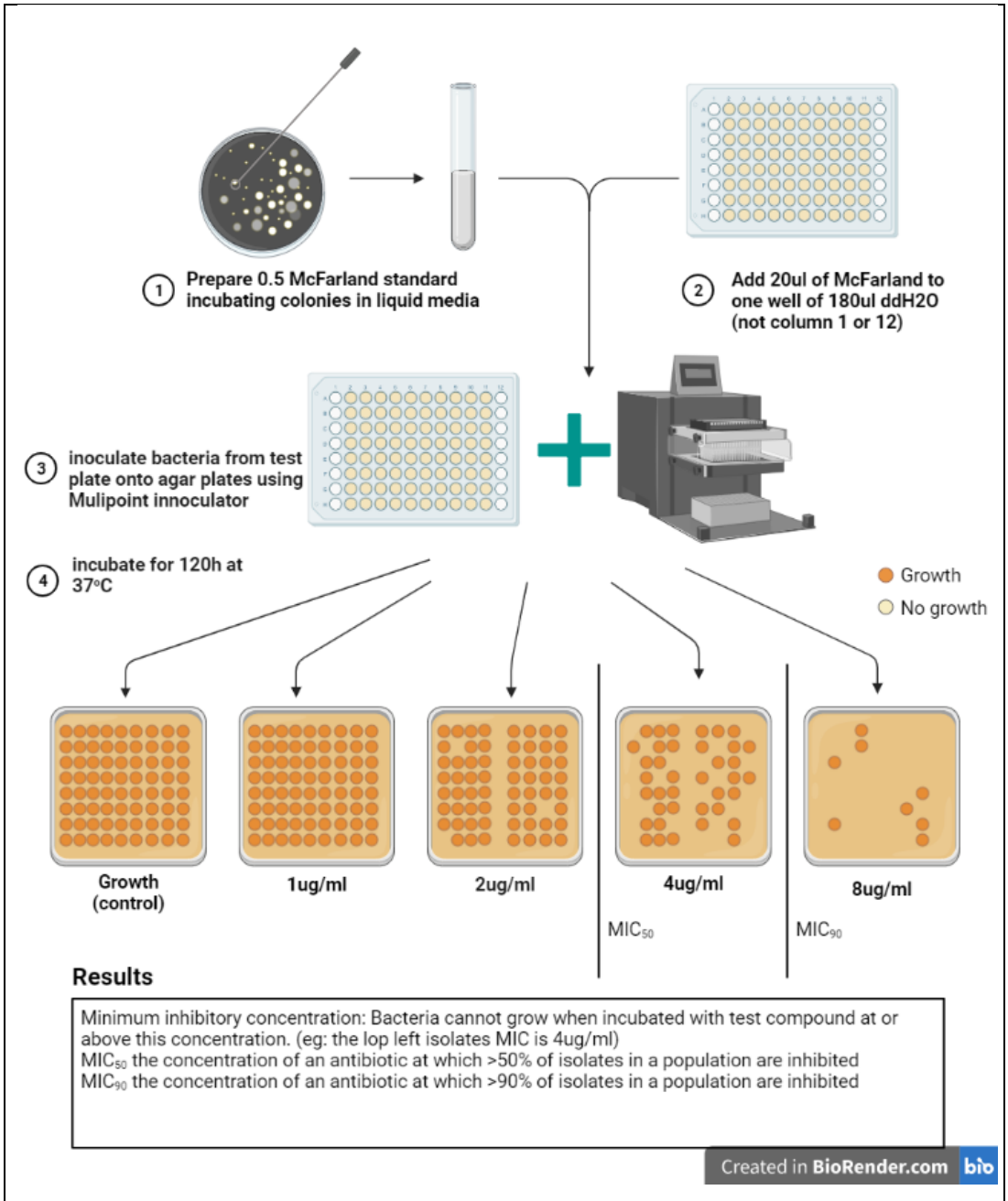


Figure 3.3 Diagrammatic protocol of the agar dilution method

This figure describes the step-by-step process used to for the agar dilution AST method made using (BioRender).

3.4.4 Alternate AST experiments

For broth microdilution MPI (Multi Point Inoculation) experiments, antibiotics were mixed with buffered yeast extract broth containing 0.1% a-ketoglutarate (BYE-α) and 200µL was placed into each well of a sterile 96-well plate.

These were then inoculated as above, with the caveat of an increased risk of carryover from broth: therefore, a new inoculation plate was used for each antimicrobial range and inoculation was always carried out from lowest to highest concentration. Pins were sterilised between antibiotic range cohorts for 15mins in 80% ethanol.

No AST results were compared to a EUCAST guideline (as would have been done for bacteria such as *E. coli* or *Klebsiella* spp.) as none have been published for *Legionellae*. Instead, they were compared with other publications that used broth microdilution. The number of isolates that were screened in this project allowed for the determination of cut off points for isolates of decreased susceptibility.

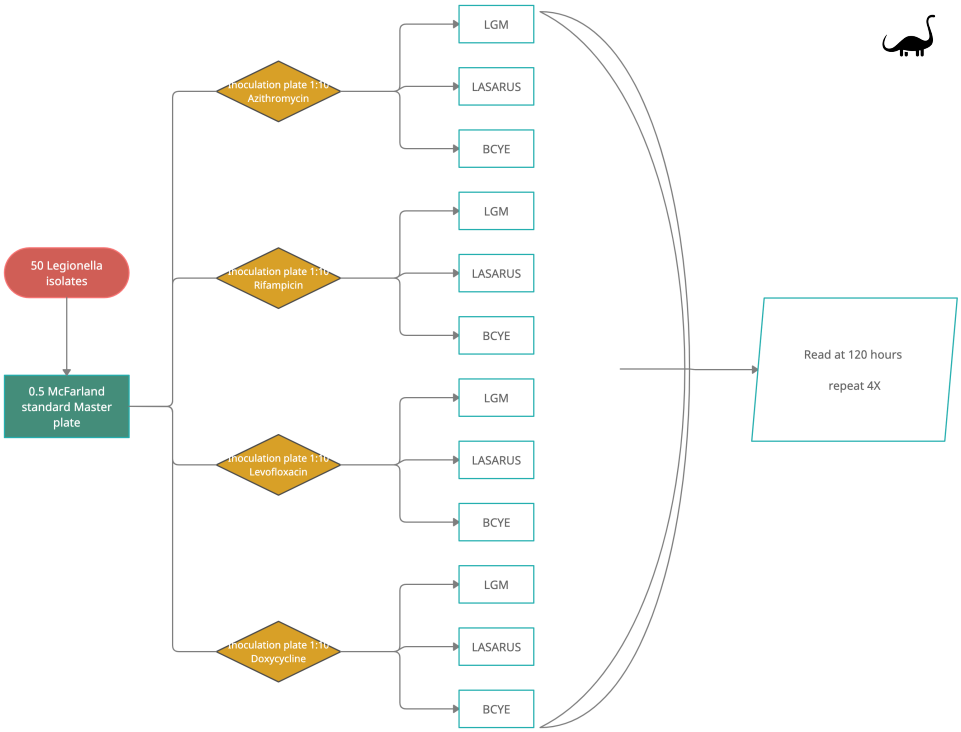


Figure 3.4 Workflow for the agar comparison method

3.4.5 Non-traditional agars for Legionella experiment

For investigating the growth of *Legionella* on other non-*Legionella* agar plates each plate was prepared in square plates, inoculated with a test panel of 80 *Legionella pneumophila* isolates, incubated in a humidified chamber at 37°C for ten days.

3.4.6 Investigation of BCYE-α VS BCY-α

Media variations for a comparison of growth potential between BCYE-α and BCY-α is listed in Table 13

Table 13 Solid *Legionella* media, without activated charcoal

Reagent's g/L	**BCYE-α**	**BYE-α**
Yeast extract	10	10
ACES buffer	10	10
KOH 1N	2.8	2.8
Agar	12	12
Purified water	To 1 litre	To 1 litre
Ferric pyrophosphate	0.25	0.25
L-Cysteine	0.4	0.4
Alpha Ketoglutarate	1	1
Activated charcoal	2	none

3.5 Illumina Sequencing

Optimisation of the genomics workflow for Illumina Miseq, key areas of note with regards to method development have been included in this section.

3.5.1 DNA extraction

Whole bacterial genome extraction was carried out using 10µL of bacteria culture from a 120-hour growth plate. Suspended in ddH_2O and centrifuged at 10,000 RPM for 5mins, supernatant removed and loaded onto the automated QIAcube platform (QIAGEN), run with the QIAamp DNA Mini Kit (QIAGEN, Germany), with an additional RNAse step.

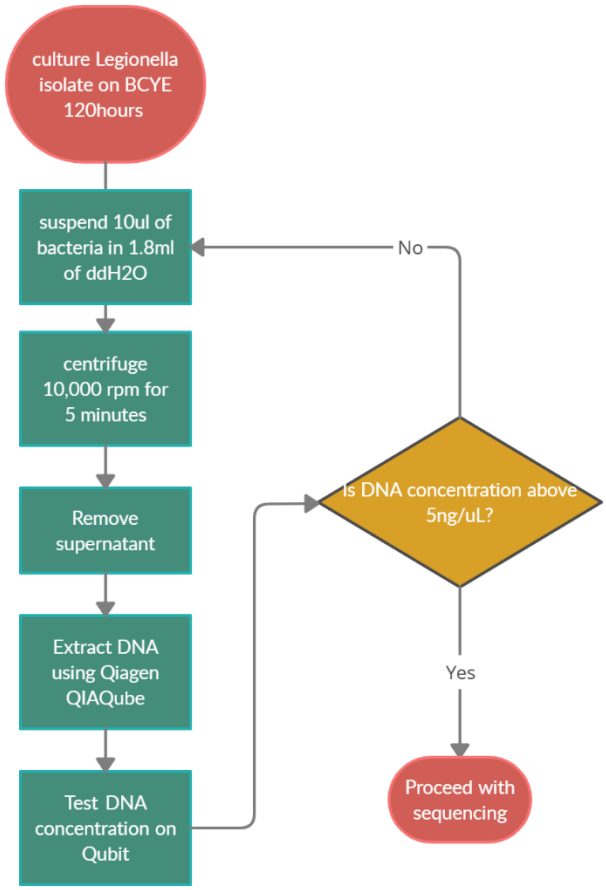


Figure 3.5 Workflow of DNA extraction protocol

DNA extraction workflow for the preparation of DNA for WGS from bacterial plate to confirmation of successful DNA (made using Creately)

3.6 DNA quantification

Genomic DNA was quantified using the Qubit fluorometer 4.0 and the dsDNA 1X Kit (Thermo Fisher Scientific). The 1X kit is a preprepared mix of buffer and dye that was used to reduce laboratory wastage as well as increase speed of use when compared to the dsDNA narrow range kit.

Two control tubes were initially set up using 190µL of 1X dye in each with 10µL of standard 1 and standard 2. (These are 10ng/µL and negative controls for the experiments). Sealed and vortexed for 2-3 seconds, left for 2mins then ran as standards on the machine. Running the standards each time is essential to ensure accurate results. The samples were analysed by adding 198µL of 1X dye to a 0.5mL eppendorf and 2µL of the sample DNA extract, vortexed for 2-3 seconds and leaving for 2mins and run through the Qubit 4.0 (ThermoFisher, USA).

Quantification had been attempted using the nanodrop, however the inaccuracies with this machine outweighed that advantage of a 260/280 ratio. Optimising the protocol by changing from a nanodrop to a Qubit reduced the number of failed isolates in a run as well as improving the sequence distribution within a run. The increased cost of Qubit reagents was offset quickly through the reduction in failed isolates within a run (~£65/isolate). Error rates in nanodrop have previously been reported (Masago et al. 2021). These form part a series of changes made to the Illumina protocol the efficacy of which can be seen in Figure 3.6 on page 89.

3.7 Genomic library preparation

3.7.1 Introduction

Genomic libraries were prepared using Nextera XT v2 (Illumina, USA), with a bead-based normalization, following manufacturer guidelines. Paired end WGS was performed on an Illumina MiSeq using the v3 chemistry to generate fragment lengths up to 300 bp (600 cycles).

3.7.2 DNA Preparation

DNA was normalised to 0.2ng/mL. This was done by adding the Qubit value (DNA concentration) along with the sample ID to the 'DNA normalisation sheet' as seen in Table 14. From this I created a 0.2ng/µL concentration plate by adding volume of DNA in µL to volume of mgH_2O in µL. Sample four shows the formulae. The full plate was then mixed with a multichannel and spun down to remove bubbles.

Table 14 Template DNA normalisation sheet
The fourth row shows the volume of DNA and water needed to obtain 100µL at 0.2ng/mL

	Input_DNA_date_indecies				
	Sample_name	Concentration (ng/µl)		DNA	mgH_2O
1	DNA sample 1	20	A1	1.0	99.0
2	DNA Sample 2	15.2	B1	1.3	98.7
3	DNA Sample 3	5	C1	4.0	96.0
4	DNA Sample 4		D1	=20/C1	=100-E6

Once finished, an additional QC (quality Control) step was added, in which each sample is Qubit quantified again to ensure 0.2ng/µL had been achieved (acceptable range (0.150 to 0.3ng/µL).

On average this additional step detected at least one isolate with too low a DNA concentration (which would therefore fail the run) as well as often identifying isolates

that had significantly more than 0.2ng/µL of DNA (by having an incorrect fragment length following enzymatic tagmentation and therefore the PCR clean up and size selection would).

3.7.3 Tagmentation, PCR and clean up

A new 96 well plate (named NTA plate) containing 5µL of TD buffer (tagmentation DNA Buffer), 2.5µL of ATM (Amplification Tagmentation Mix) and 2.5µL of (recently mixed and spun down) DNA at 0.2ng/µL (as made in 3.7.2 DNA Preparation) was made.

This was then mixed, spun down and sealed (using a PCR seal), and placed in a pre-heated thermocycler at 55°C for 5mins. Then, 2.5µL of NT (Neutralise Tegument Buffer) was added, mixed and left for 5mins at RT to inhibit further tagmentation of DNA.

7.5µL of NPM (Nextera PCR Master Mix) was added to the NTA plate, a different 2.5µL of white index was added to each well of the 96well plate, and a different 2.5µL of orange to each of the columns (this ensured that each cell gets a unique combination of orange and white index bound to its fragmented DNA, allowing for demultiplexing post sequencing). Mixed, sealed, spun, and run through the PCR conditions in Table 15.

Table 15 PCR cycle for Illumina MiSeq

Temperature ^{o}C	Time (seconds)	Repetitions
72	180	1
95	30	1
95	10	12
55	30	
72	30	
72	300	1
10	∞	1

In the Illumina protocol this was considered a safe stopping point at which the DNA could be placed into a fridge and kept for up to two weeks, I have found that even an overnight delay significantly decreased the overall cluster density of the run thereby significantly reducing the quantity of data output.

3.7.4 PCR Clean up and Library normalisation

11.5µL of SPRI (Solid Phase Reversible Immobilization) beads was added to each well of a 0.8mL MIDI plate labelled 'Clean Amplified Plate'.

SPRI beads are pre-warmed to 30^{o}C as otherwise yield from is significantly reduced. To ensure a uniform sequence the beads must be well diffused in the liquid, achieved by vortexing for at least 30 seconds.

Reducing the volume of SPRI beads from 12.5µL to 11.5µL improved the quality of our read as it reduced the number of small fragments that were selected and kept within the DNA preparation. Thereby improving the quality of the DNA being sequenced.

A graphical comparison of metrics before and after all changes were made is shown in Figure 3.6, which shows an early poor quality (A) run followed by an improved run (B) after some of the changes were made and subsequently a high-quality run (C) with good sample distribution shown

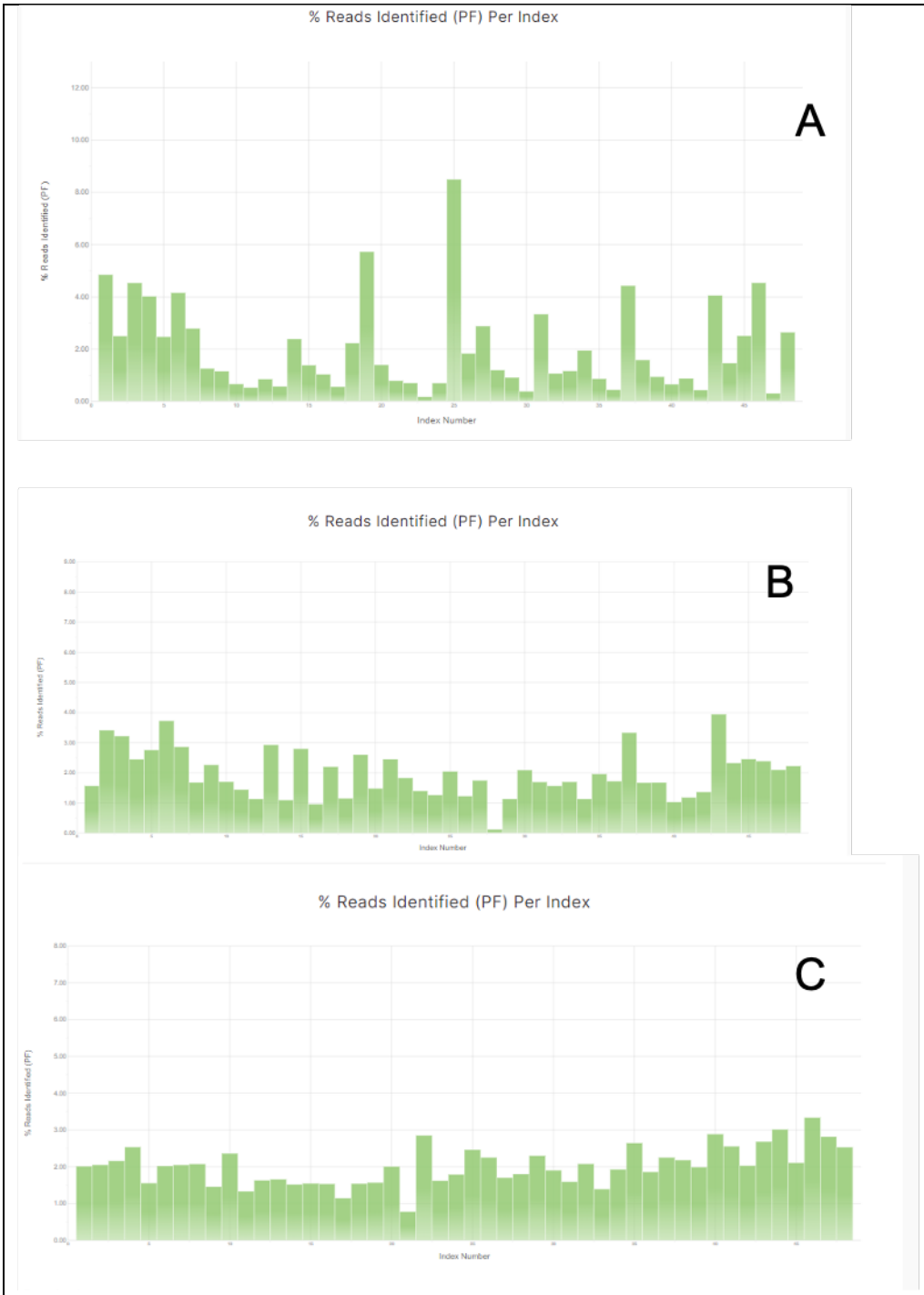


Figure 3.6 Index read per sample for three MiSeq runs
Highlighting A: a low-quality run, B: a medium quality run and C a high-quality run. Information from BaseSpace.

Then sealed and spun (pulsed (10seconds at 1,000rpm), then shaken on a VWR DMS-2500 High speed Microplate shaker (Figure 3.7) at 1,800rpm for 2mins.

Figure 3.7 A VWR DMS-2500 High speed Microplate shaker

The tubes were left at RT for 5mins and placed on a neodymium magnetic stand for 2mins. The supernatant was removed, (tips which are flexible are essential here to ensure that the beads are at the bottom edges of the well are not also eluted). An 80% ethanol wash (100µl per sample) was performed into well, twice. Once eluted the plate was left to dry for 15mins.

Eluted into 25µL of RSB (Resuspension Buffer) shaken for 2mins at 1,800rpm, incubated at RT for 2mins, placed on a magnetic plate for 2mins. Eluted 20µL of the supernatant into a new 96well plate labelled CAN.

At this point DNA concentrations were re-calculated and recorded in Table 14.

3.7.5 Library normalisation

A new MIDI plate labelled LNP (Library Normalisation Plate), containing 22.5µL of a combination of LNA1 and LNB1 to each well (at a ratio of 5.5:1 by mixing 2,200µL and 400µL of LNA1 and LNB1 respectively for 96 samples) was made.

10µL of sample from the CAN plate was added to each respective well. The LNP plate was shaken at 1,800rpm for 30mins. The 30 minute incubation step is crucial for proper library normalisation; longer or shorter shaking periods can affect library representation and cluster density.

The LNP plate was placed in the magnetic stand for 2mins, the supernatant was discarded and 22.5µL of LNW1 was added into each well of the LNP plate, sealed, spun down and shaken for 5mins at 1,800rpm placed on the magnetic stand, the supernatant was discarded and the LNW1 wash repeated.

A fresh solution of 0.1M NaOH was then made. (10M NaOH solution: 4950µL MGH_2O + 50µl 10M NaOH). It is essential that this is prepared fresh magic pixies that make this protocol work get cross and the run inevitably fails. 15µL of NaOH was added into each well and shaken for 5mins at 1,800rpm Placed onto a magnetic rack after 2mins 10µL of the supernatant was transferred into a 96well plate containing 10µL of LNS1. Then spun for 1min at 1,000rpm.

3.7.6 Loading MiSeq

All samples were pooled into a 1.5mL eppendorf labelled PAL (Pooled Amplicon Library), 24µL of PAL were transferred to a 1.5mL eppendorf labelled DAL - containing 576µL of HT1. 100 µL of DAL was discarded and 10µL of 20pmol PhiX was added. The DAL was then topped up with HT1 to equal 600µL.

DAL was the incubated at 96°C for 150 seconds inverted then place in an ice bath. The sample was then added to a thawed MiSeq reagent cartridge which was then tapped down. The reagent cartridge was then loaded onto the MiSeq and on-screen instructions were followed.

3.8 Oxford Nanopore Technologies (MinION)

3.8.1 ONT methods introduction

Two different protocols were used for ONT library preparation, these differed based on the starting sample with one being BAL samples and one being a QiaCube extracted DNA. One involved PCR and one was PCR free.

3.8.2 MinION

First the flow cells were QCd to ensure there were enough pores for the run (minimum of 800 for 12 samples).

The DNA (extracted on the Qiacube as in 3.5.1), was combined with the genomic DNA at a ratio of 1:1 to SPRI beads (using a pipette to estimate the total volume of gDNA) on a MIDI plate and shaken on the CAPP shaker for 2mins at 1800rpm, left at RT for 2mins, placed on the magnet for 2mins then the supernatant was discarded. Two ethanol washes were performed as with the Illumina prep (on the magnet add 200µL of 80% ethanol, immediately remove & repeat). Once the second ethanol wash had been removed, the plate was incubated for a maximum of 5mins to air dry (without over drying as with the Illumina prep excessive drying would cause the beads to fail to release the DNA). Between 15-30µL of MGW was added to the dried beads. Shaken for 2mins at 1800rpm and placed on the magnet. 2µL of the eluent was removed for qubit measurement using the BR kit. The remaining sample was then adjusted to between 40-60 ng/µL using MGW. In PCR tubes each containing 7.5µL of the normalised gDNA was added to 2.5µL of fragmentation mix barcode (using a different barcode for each sample). This was mixed gently by flicking the tube and spun down.

In a thermal cycler, the tubes were incubated at 30°C for 1min and then at 80°C for 1min then placed on ice for 1min. The samples were then pooled into a 500µL eppendorf. The pooled mix was then added to SPRI beads at a 1:1 ratio of DNA and rotated gently for 5mins. This was then placed into a magnetic stand and for at least 5mins. Two ethanol washes were performed (on the magnet add 200µL of 80% ethanol, immediately remove & repeat). Once the beads were dry, 15µL of molecular grade water, was added. This mix was then shaken for 2mins at 1800rpm and left at RT for 2mins, then placed onto the magnet until clear (around 5mins). 2µL was quantified using the Qubit, leaving at least 10µL. 10µL was then transferred to a 0.5mL eppendorf containing 1µL of RAP. Mixed gently and incubated at RT for 30mins (this step is not included in the MinION protocol but as with the NaOH failing to carry it out will irritate the pixies), this could then be stored on ice until ready for loading preparation.

3.8.3 Loading the flow cell Preparation

The MinION lid and the sliding port were opened so that the priming port was visible. That a continuous buffer permeated the priming port was ensured by removing a small volume of the buffer to ensure there were no bubbles as these could damage the pores. 800µL of mixtures of FLT and FLB were loaded into the flowcell priming port. This was then left for 5mins during which time the DNA was prepared as per Table 16.

Table 16 Final loading reagents for a MinION run

reagent	Volume µL
SQB	34
LB (well mixed)	25.5
MGW	4.5
DNA library	11

Total	75	

After 5mins the SpotON sample port cover was lifted and 200µL of the priming mix (FLB+FLT) was loaded into the flow cell via the priming port (not the SpotON sample port), The final DNA Library was then mixed and via the SpotON sample port in a dropwise fashion. All ports and the lid of the MinION were then closed. And using the MinKNOW GUI, following on-screen instructions, selecting SQK-RBK004 as the kit. All samples were run for 72 hours.

3.9 Bioinformatics

3.9.1 Illumina Miseq Bioinformatics

Bioinformatics analysis was performed using a high-performance computing cluster at Cardiff University (ARRCA) and CLIMB (Connor et al. 2016). Paired-end reads (FASTQ) were subjected to quality control checks before down-stream analysis. Trimgalore (v0.4.3) (Krueger 2017) was used to remove the Nextera adapter sequences and low-quality bases. Reports before and after read trimming were generated using fastQC (v0.11.2) (Andrews 2019) and collated using MultiQC (v1.7) (Ewels et al. 2016). The mean read length and number of sequences provided on the MultiQC reports was used to determine sequencing coverage. Paired-end reads were overlapped using Flash (v1.2.11) (Magoč and Salzberg 2011) and assembled into contigs using SPAdes (v3.9.0) (Bankevich et al. 2012). The trimmed FASTQ reads were mapped to the contigs using BWA (v.0.7.15) (Li and Durbin 2009) and samtools (v1.3.1) (Li et al. 2009). Pilon (v1.22) (Walker et al. 2014) was used to assess any misassembles/errors in base calling in the resulting mapped BAM file. The assembly pipeline (which includes BWA, samtools, SPAdes, Pilon) shovill v.0.9.0 was used (Github URL https://github.com/tseemann/shovill). Final genome assembly metrics were generated using quast (v.2.1) (Gurevich et al. 2013). MLST, antibiotic resistance, virulence and plasmid genomic profiles were characterised using srst2 (v0.2.0) (Inouye et al. 2014) and associated databases: abricate, ARG-ANNOT (Gupta et al. 2014), PlasmidFinder (Carattoli et al. 2014) and VFDB (Chen et al. 2005). Genomes were identified using PathogenWatch (a web application) and annotated using Prokka (v1.12) (Seemann 2014). Further analysis was carried out using Geneious sequence analysis software (version R10; BioMatters ltd., New Zealand).

3.9.2 MinION Bioinformatics

Long reads were base called using Guppy within Minknow, demultiplexed using Porechop (v0.2.4) (Wick et al. 2017) and assembled against corresponding short reads generated from the Illumina MiSeq using Unicycler (v0.4.7) (Wick et al. 2017) with default parameters. The hybrid assembly was assessed using quast (v5.0.2) and ABRicate (v0.9.7) (Seemann 2019) (>98% coverage and identity) was used to search for detect antimicrobial resistance genes (ARG). The MobileElementFinder database (v1.0.2) was downloaded and ABRicate (Seemann 2019) was used to search for the mobile genetic elements (MGE) genomic context of ARG. Legsta (v0.5.1) was used for sequence-based typing of *Legionella pneumophila.*

3.9.3 Bioinformatics tree

Kraken2 was used to evaluate the species and check the sequences for contamination or unclassified reads. A core genome alignment of 40 *Legionella* isolates was made using Prokka annotations and Roary v3.12.0 (Page et al. 2015). A core genome tree was made using IQtree v2.0 (Nguyen et al. 2015). The phylogenetic tree was annotated in iToL v6 (Letunic and Bork 2021).

3.10 Statistical analysis

All statistical analysis was carried out using Prism GraphPad version 7.03 and statistical significance was set at p=<0.05. One-way Anova was run with Bonferroni correction, when assuming multiple points, assuming normal distribution parametric analysis. A paired T-test was run if the data was comparable.

3.11 MALDI-ToF-MS

MALDI-ToF-MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry) (henceforth referred to as MALDI) was used to identify *Legionella* species as well as contaminants using a Microflex LT MALDI from Bruker, UK.

Samples were grown fresh to ensure that pure single colonies were available as per 3.3, single colonies were then picked using a cocktail stick and a small quantity of bacteria was placed onto a well of a MALDI plate, using the same cocktail stick (without applying more bacteria) a second spot was filled. (This is done to ensure a wide enough range of bacterial loads for a positive identification).

1µL of MALDI matrix (α-Cyano-4-hydroxycinnamic acid (HCCA) (Sigma, UK) was applied and left to dry for 15mins. This was then processed by SACU (Specialist Antimicrobial Chemotherapy Unit at the University Heath Hospital Wales).

3.12 qPCR

qPCR (quantitative PCR) was used to detect and quantify *Legionella* DNA. Using a CFX-96 BioRad, the fluorophore used was ROX. Figure 3.8 and Figure 3.9 show the full in-house developed plasmid used for quantification, the *mip* gene target and primer sequences respectively.

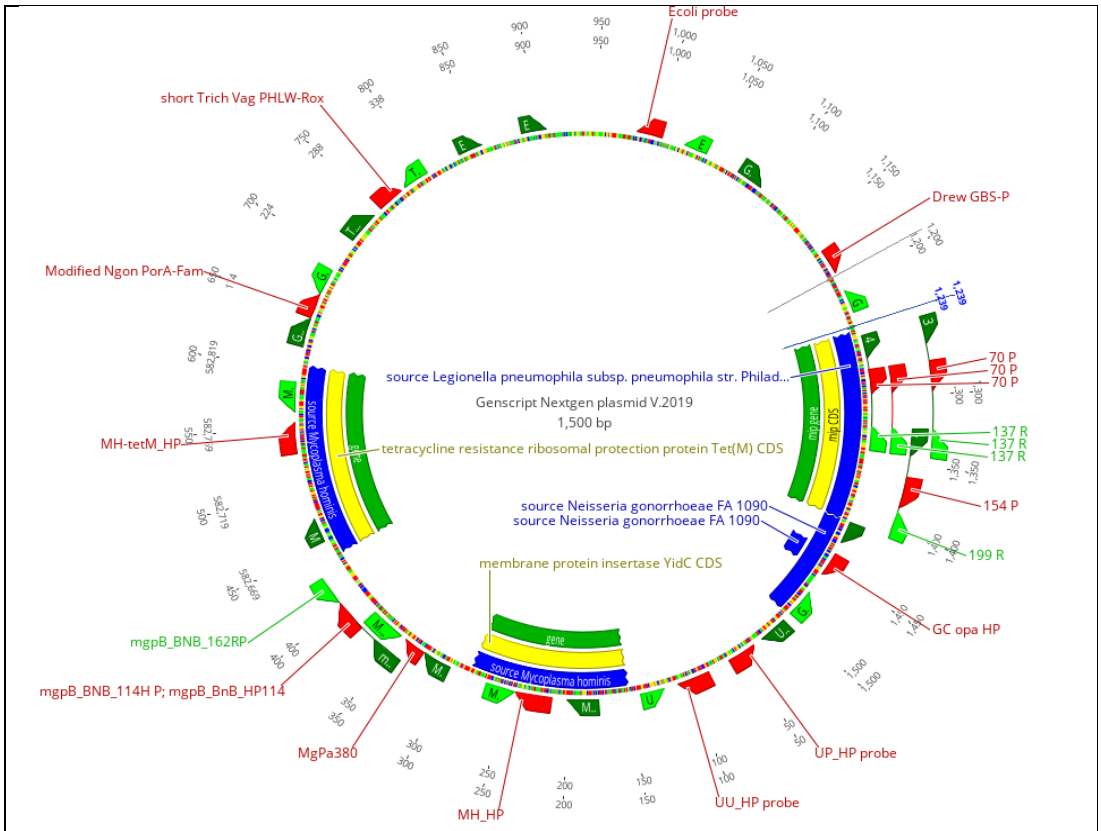


Figure 3.8 Circularised plasmid with qPCR target for quantification of *Legionella*

View of the inhouse developed circularised plasmid containing targets for *mip* (*Legionella*) as well as other targets.

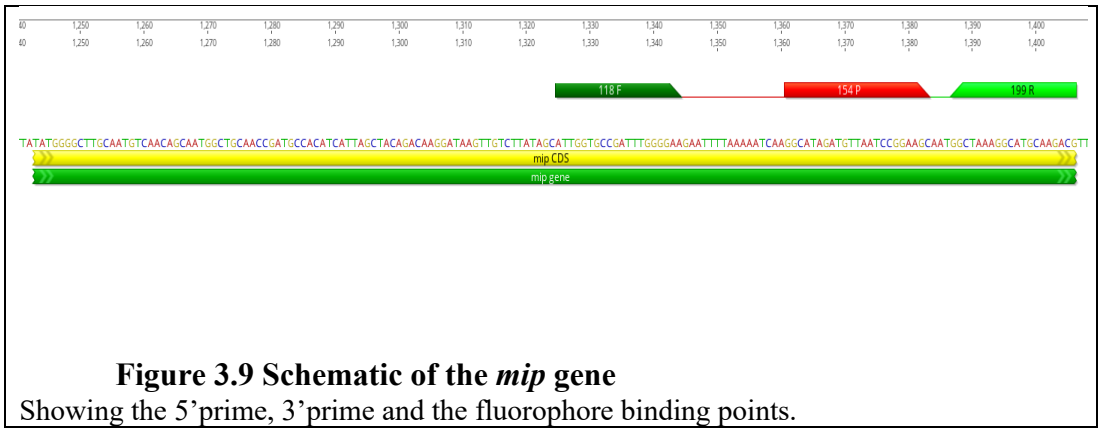


Figure 3.9 Schematic of the *mip* gene

Showing the 5'prime, 3'prime and the fluorophore binding points.

Table 17 MIP Primer sequences

Primers	5'-3' sequence
MIP-FP	ATTGGTGCCGATTTGGGGAA
MIP RP	CGTCTTGCATGCCTTTAGCC
MIP Probe	GGCATAGATGTTAATCCGGAAGC

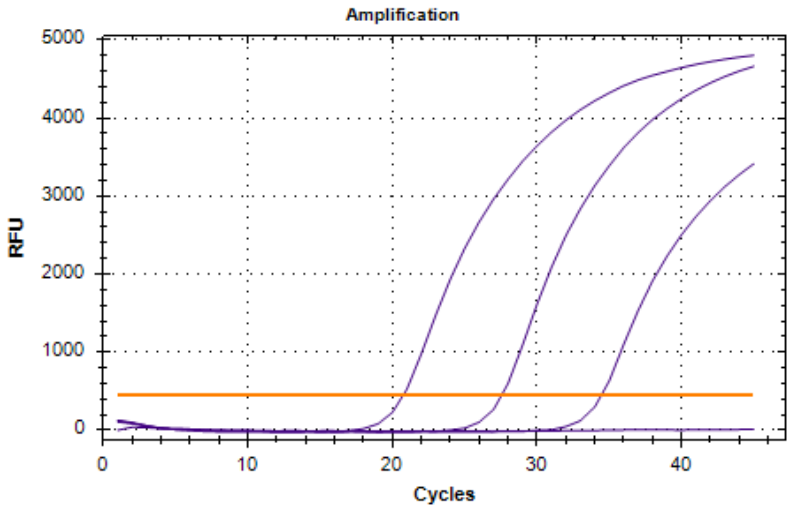


Figure 3.10 A standard curve of *mip* showing, showing 10^6, 10^4 and 10^2 copies of *mip*.

A standard curve to measure the genomic copy number was created (Figure 3.10) to compare 1,000,000, 10,000 and 100 copies of the target in a plasmid to the bacterial lysates.

Colonies were boil-lysed for 5mins in sterile water and then subjected to qPCR analysis. The presence or absence of *Legionella* was determined as having a Cq of below 35 cycles.

4 Results chapter 1:

4.1 Introduction

As discussed in the primary introduction, growing *Legionella* has always been problematic. It delayed the initial discovery of the Philadelphia pathogen by several weeks as it could not be grown on the traditional agars available at the time. Only after it was treated as a virus was it successfully cultured in eggs. It was discovered that toxic metabolites formed during the autoclaving process of agar inhibited *Legionella* growth, the addition of activated charcoal resolved this problem. However, the chelating effects of activated charcoal are not limited to agar toxins but would be equally effective in the chelation of other chemicals, including antibiotics and other antimicrobial compounds added to the media to enable determination of minimum inhibitory concentrations. This was reinforced with an expansive literature review (Portal et al. 2021b) which highlighted the significantly elevated MICs found when using BCYE-α containing activated charcoal compared to liquid broths-based methods (the later does not require activated charcoal) (Ruckdeschel and Dalhoff 1999; Bruin et al. 2012).

For MIC screening on a large scale, liquid-based methods are both time consuming and inefficient. An agar-based approach was essential, particularly to identify non-*Legionella* contamination of samples. A media was required with the same growth properties as BCYE-α whilst lacking the chelating effects of activated charcoal. Ideally, an improved *Legionella* media would:

1. Lack a chelating compound such as activated charcoal.
2. Have similar or better growth properties as compared to BCYE-α.
3. Support growth of all *Legionella* spp.

4. Have similar production costs relative to BCYE-α.
5. Lack a complex manufacture process.
6. Facilitate bacterial growth observation without the requirement to remove the lid (improved safety and enable automated plate reading during use).
7. Allow observation of differential colony pigmentation to improve bacterial species discrimination.

4.2 Critical evaluation of the alternative media

An extensive literature search performed as part of a patent application highlighted several possible alternatives, which were evaluated for large scale screening, ease of use, ease of manufacture, cost, and other important characteristics above. The most viable of these alternatives are presented and assessed here

4.2.1 Buffered Starch Yeast Extract (Saito, et al. 1985):

Saito, *et al*. in 1985 investigated a charcoal-free medium for growing *Legionella* for testing MICs. They had noted that activated charcoal would inhibit MIC determination and devised an alternative media called BSYE (Buffered Starch Yeast Extract). The medium contained yeast extract, ACES buffer, potassium hydroxide, sodium L-glutamate, starch, agar, purified water, L-cysteine, and ferric pyrophosphate. See Table 18 for exact formula compared to BCYE-α).

Table 18 Component comparison between BCYE-α and BSYE

Reagent g/L	BCYE-α	BSYE
Yeast extract	10	10
ACES buffer	10	10
KOH	2.8	2.5
sodium L-glutamate	n/a	5
Agar	12	15
Purified water	To 1 litre	To 1 litre
Ferric pyrophosphate	0.25	0.25
L-Cysteine	0.4	0.4
Activated charcoal	2	None
Alpha Ketoglutarate	1	None
Soluble starch	None	15
pH	6.7-6.9	Not revealed

The primary difference between BSYE and BCYE-α is that activated charcoal has been substituted for starch (i.e. starch was used as an alternative chelator to activated

charcoal), with the expected advantage that MIC results would not be adversely impacted (Saito et al. 1985). (MIC results are shown in Appendix A).

This methodology was later compared to BCYE-α by Pendland, *et al.* 1997 as a comparison between activated charcoal and starch-based media and the effect on growth (Pendland et al. 1997). This subsequent report found that *Legionella* species yielded better growth on BCYE-α agar than BSYE, and that the reported growth rates produced by Saito *et al* were unreproducible. Pendland, *et al* concluded that BCYE-α was far superior to BSYE for testing the susceptibility of *Legionella* to antimicrobials whilst acknowledging the artificially elevated MICs. These latter authors speculated that growth on BSYE was so limited it in effect artificially lowered the tested MIC values due to a lack of bacterial biomass (Pendland et al. 1997). Thus, starch as a replacement for activated charcoal in the media was discounted and this media was excluded from my investigation based on its poor growth as compared to BCYE-α.

4.2.2 BioMérieux T1, T2 & T3 Silica (patent number US 8,709,746 B2):

A French patent submitted in 2014 (patent identifier: US 8,709,746 B2) (Cellier et al, 2014) supported by BioMérieux proposed the use of polar silica in which a siliceous substrate to replace activated charcoal.

Table 19 BCYE-α compared to BioMerieux T, T1 and T2

Reagent g/L	BCYE-α	Biomerieux T	Biomerieux T1	Biomerieux T2
Yeast extract	10	10	10	10
ACES buffer	10	4	4	4
KOH	2.8	1.65	1.65	1.65
Agar	12	17	None	17
Purified water	To 1 litre	To 1 litre	To 1 litre	To 1 litre
Ferric pyrophosphate	0.25	0.25	0.25	0.25
L-Cysteine	0.4	0.4	0.4	0.4
Alpha Ketoglutarate	1	1	1	1
Activated charcoal	2	None	None	None
Glycine	None	3	3	3
Glutathione	None	5	5	5
Octadecyl-silica	None	1	1	1
Gelrite	None	None	17	None
Sepiolite	None	None	None	5

There are multiple factors with this media, which preclude its inclusion for routine use. The cost associated with this media is high and required novel components of limited availability; Octadecyl-silica is both expensive and difficult to procure and only available to purchase in quantities in excess of 5Kg. The use of a silica suspension poses further technical problems: silica suspension has similar properties to activated charcoal in that it requires continuous agitation to avoid settling out of solution and therefore is not uniform across an entire batch. In other words, without specialist manufacturing equipment, silica sedimentation whilst pouring, will result in intra-batch differences and the first and last plate poured in a research laboratory setting will have different concentrations of silica. This intra-batch diversity could also impact

altered chelating potential rendering MIC results or *Legionella* spp. growth inconsistent. Secondly, and more importantly, silica powder is a potent irritant and long-term exposure can cause silicosis (The Lancet Respiratory Medicine 2019), a respiratory inflammatory condition associated with irreversible scarring of the lung tissue and linked to induction of lung carcinoma. It therefore requires handling within chemical fume containment cupboards and thus was also discontinued as a viable alternative to BCYE-α. The interesting concept of using either gelrite or sepiolite as either an additive or a substitute to agar was also proposed in this patent application.

4.2.3 Serum albumin agar (Armon and Payment, 1990)

A report by Armon and Payment 1990 (Armon and Payment 1990) noted the primary problems associated with using a black opaque media, as well as highlighting the loss for potential chromogenic incorporation (especially the loss of the pigment/siderophore production by some *Legionella* spp. and difficulty with plaque visualisation in phage isolation experiments). They devised a novel media, LTM (*Legionella* Transparent Media), with the replacement of activated charcoal with bovine serum albumin fraction 5 (BSA-F5) at a high concentration, as well as the use of proteose peptone 3. These alternatives acted as both growth enhancer and chelator of the free fatty acids and free radical toxins released through agar autoclaving. While this media was theoretically fit for potential in *Legionella* spp. AST, it was disregarded for large scale AST testing primarily due to the prohibitive cost and low availability of BSA-F5 (around £1000/Kg). In addition, DIFCO protease peptone 3 is an expensive proprietary formulation that was not available for purchase.

Table 20 BCYE-α compared to LTM

Reagent g/L	BCYE-α	LTM	LTM MOPSO	LTM PIPES
Yeast extract	10	1	1	1
ACES buffer	10	10	None	None
KOH 1N	2.8	3.6	3.6	3.6
Agar	12	17	17	17
Purified water	To 1 litre	To 1 litre	To 1 litre	To 1 litre
Ferric pyrophosphate	0.25	0.25	0.25	0.25
L-Cysteine	0.4	0.4	0.4	0.4
Alpha Ketoglutarate	1	1	1	1
Activated charcoal	2	None	None	None
Proteose peptone 3	None	15	15	15
Bovine serum albumin Fraction V	None	100mL	100mL	100mL
MOPSO	None	None	10	None
PIPES	None	None	None	10

There were two other notions of interest from Armon and Payment 1990. Firstly, the addition of tyrosine increased the speed at which browning was visible with some *Legionella spp.* from 5-6 to 3-4 days. Secondly, less expensive buffers MOPSO (2-Hydroxy-3-morpholinopropanesulfonic acid) and PIPES (piperazine-N, N′-bis (2-ethanesulfonic acid)) were shown to have similar growth potential to ACES buffer, which was more costly. Unfortunately, validation of this data has not been detailed sufficiently by Armon and Payment 1990 or in subsequent studies and I have not been able to repeat their observations. Therefore, these components were not included for development of high-throughput *Legionella* spp. AST.

4.2.4 Ryuichi endo (patent identifier:JP12059297A)

A Japanese patent submitted in 1997 (patent identifier:JP12059297A) (Endo et al, 1997) suggested the use of gellan gum as a substitute for agar removing the necessity for any chelating compound. However, the exact production is proprietary, gellan gum is expensive and hazardous in case of inhalation, skin contact, ingestion or eye contact (Gellan Gum MSDS, Gellan Gum Materials Safety Data Sheet [no date]). As the production formulation was not available to evaluate this was disregarded from further study.

4.3 Evaluation of more suitable alternatives from the literature.

4.3.1 Washed Buffered Yeast Extract (Rogers et al., 1992):

A method of physically cleaning agar so that when autoclaved the agar did not release the aforementioned toxic by-products was published by Rogers *et al* in 1992 WBYE (Washed Buffered Yeast Extract). In this protocol 20g of agar were washed five times with 5litres of ddH_2O and then used to prepare a solid culture medium in the absence of activated charcoal. This theoretically would create a solid charcoal free agar, which would not only allow *Legionella* growth but also enable growth to a similar degree as BCYE-α. However, the authors reported a 21–24% reduction in growth efficiency compared to BCYE-α agar (Rogers et al. 1993). Despite these limitations, reproduction of this method was attempted as it was inexpensive in bulk and had the potential for growth optimisation. Three attempts were undertaken to wash agar and produce a solid culture plate, but none of the resulting agar plates were fit for purpose. For example, the third attempt produced a semi-solid agar that had the consistency of a plate roughly equivalent to 0.25-0.5% agar (potentially of use for phage experiments, but incompatible with my needs). In particular, none of the WBYE attempts made an end product that appeared suitable for multi-point inoculation to enable large scale MIC testing, as the formulation would not dry and could not be inverted. One attempt was made to inoculate my standard test panel of 80 *Legionella pneumophila* isolates onto the semi solid plate and incubate them without inversion. Results were unsatisfactory for two reasons: the inoculum spread easily over respective boundaries on the inoculation plate and carry over of the media back to the inoculation reservoir was observed when using multi-point inoculation resulting in cross-contamination of the equipment. Furthermore, non-*Legionella* spp. contamination on this formulation

spread rapidly throughout the agar rending results unreadable. Furthermore, the subsequent growth of inoculated *Legionella* species was much lower than the 21-24% reduction reported by the authors and often yielded negligible distinguishable *Legionella* spp. growth.

This media formulation was also impractical for routine use as it required filtering enormous quantities of water through 20g of agar that would not be practical for routine manufacturing.

4.3.2 Investigation of traditional non-Legionella cultivating media:

To investigate whether *Legionella* species would grow on conventional media it was important to review growth on a range of specialised formulations. The primary investigations into media development were carried out in the 1980s and subsequent improvements to formulation of media may have occurred over time with regards to potential toxic metabolites which may have been removed from traditional agars in the intervening decades. The table below shows a list of the media tested in this study.

Table 21 Common non-*Legionella* agar growth media

Agar	Supplier
Mueller Hinton	Oxoid
MacConkey's	Oxoid
CBA (Columbia Blood Agar)	Oxoid
Chocolate blood agar	Oxoid
UTI chromogenic	Liofilchem
Lysogeny Broth (LB) agar	Oxoid
Tryptic soy agar (TSA)	Oxoid
Xylose Lysine Deoxycholate agar (XLD)	Oxoid
Cystine lactose electrolyte deficient (CLED)	Oxoid

No trace of *Legionella* spp. growth could be detected. This was in spite of my expectation to see any trace of growth on media for fastidious organisms (blood/chocolate agars). An extensive interrogation of internet documentation on

growth of *Legionella* (not limited to peer-reviewed publications) revealed https://www.cdc.gov/legionella/labs/procedures-manual.htmL a 2005 CDC Laboratory Guidance for Processing Environmental Samples. Footnotes within this document detailed avoiding the use NaOH as a pH adjusting agent but recommending the alternative KOH as NaOH had been shown to inhibit growth. Unfortunately, data supporting this claim or reference to a published source substantiating this observation was not detailed.

I then adopted the sodium-inhibition hypothesis, and scrutiny of the Table 21 agar formulations revealed each invariably had a high sodium concentration. However, it became clear that in the absence of a suitable existing charcoal-free media (commercial or in the literature), that a novel formulation was required.

4.4 Creation of LASARUS (Legionella Antimicrobial Susceptibility and Resistance Universal Screening) medium.

Pasculle first created a medium which could grow *Legionella* which was BCYE a charcoal supplemented buffered yeast extract (Pasculle et al. 1980). This media was then modified by Eldstein's through the addition of alpha-ketoglutarate. This media is inexpensive and simple to make for the growth of a complex intracellular organism as well as requiring no environmental supplementation such as oxygen or CO_2. Serendipitous or otherwise, addition of activated charcoal to offset the presence of agar toxins released by autoclaving has dominated the field of *Legionella* spp. growth for decades and all potential alternatives (above) have failed to achieve equivalence, let alone advancement for culture suitable to large scale AST testing.

4.4.1 Attempts at removing Charcoal from BCYE-α for Legionella growth.

Legionellae are complex bacteria. Their primary energy source is serine and threonine over an organic substrate (George et al. 1980; Oliva et al. 2018) and they have an absolute requirement for amino acids: Arg, Cys, Ile, Leu, Met, Thr, Val, Ser, Pro, and Phe (Oliva et al. 2018). Dogma suggests that replication in the "wild" only occurs via a parasitic existence within amoeba or macrophages (Oliva et al. 2018). Creating a novel charcoal-free media using BCYE-α as a baseline seemed logical. In particular, conserved inclusion of L-cysteine, alpha-ketoglutarate, the original (if not unusual) buffer and end product pH. Therefore, my initial Buffered Yeast Extract (BYE-α) formulation is listed in Table 13 and can be found in methods. A growth comparison between BYE-α and BCYE-α was carried out with my standard test panel of 80

Legionella pneumophila isolates. While BCYE-α yielded good growth on each of the three replicates tested, BYE-α yielded no growth as expected.

4.4.2 Evaluating non-charcoal replacements to absorb agar toxins and buffers alterations.

Using experience gained from growing other fastidious organism, which require substantial media supplementation to ensure growth, such as *Ureaplasma* and *Mycoplasma*. The addition of heat inactivated sheep serum as both a chelator of toxins and growth promotor was suggested. Inclusion of 20% heat inactivated filter sterilised sheep serum was made in BYE-α (HSBYE Table 22). ACES buffer is particularly expensive and difficult to source; therefore, a systematic comparison of formulation was tested where only one variable was altered (Table 22) tested as per 3.4.3.

Table 22 Varying formulation of novel *Legionella* AST medium.

	Formulation HSBYE										
Components	A	B	C	D	E	F	G	H	I	J	K
Agar (g)	1	1	1	1	1	1	1	1	1	1	1
H_2O (mL)	69	69	69	59	59	69	89	80	39	69	69
10% yeast (mL)	10	10	10	10	10	10	10	10	10	10	10
Sheep Serum (mL)	10	10	10	10	10	10	-	10	10	10	10
4g/1000mL Cysteine (mL)	1	1	1	1	1	1	1	-	1	1	1
Legionella supplement(mL)	10	-	-	-	-	-	-	-	-	-	-
Alpha ketoglutarate (0.1%)	-	10	10	10	10	10	10	10	10	10	10
0.25% $Fe_4(P_2O_7)_3$ (mL)	-	10	10	10	10	10	10	10	10	10	10
0.5M MOPS (mL)	-	-	-	-	10	10	-	-	10	10	-
0.5M Compound E (mL)	-	-	-	-	-	-	-	10	-	-	-
0.5M TRIS (mL)	-	-	10	-	-	-	-	-	-	-	-
0.5M KHPO4 (mL)	-	-	-	10	-	-	-	-	-	-	-
0.5M ACES (mL)	-	-	-	-	-	-	-	-	-	-	10

Strong and universal growth was recorded in test A, whereas no noticeable growth was observed for formulations B-J after five days. However, formulation K (which is essentially a replication of the components of the commercial Sigma *Legionella* growth supplement) produced growth after five days at 37°C relative to formulation A. Due to its reduced cost compared to formulation A, formulation K became my novel media of choice. This formulation forms the basis of the patent and from this point forward will be referred to as LASARUS (***Legionella* Antimicrobial Susceptibility and Resistance Universal Screening**).

4.4.3 Comparison of Legionella growth on LASARUS and BCYE-α.

Rather than assessing these using my standard 80 isolate test panel as previously performed, I wanted a more quantitative comparison for supporting growth. Therefore, comparison plates were inoculated with a replicate serial 2-fold dilution series of bacterial suspensions. To evaluate a range of serogroups, the initial experiments (conducted in triplicate) used the NCTC strains of differing serogroups outlined in Table 23 with the growth comparison results in the graph Figure 4.1.

Table 23 NCTC *L. pneumophila* strains used for comparison of BCYE-α and LASARUS growth

NCTC number	Strain designation	Serogroup
11230	Togus-1	2
11232	Bloomingtion-2	3
11233	Los Angeles-1	4
11406	Chicago-2	6
11984	Chicago-8	7
11985	Concord-3	8
12000	Leiden-1	10
12006	Benidorm 030E	1
12008	Olda	1
12179	797-PA-H	11
12181		13
12286	Knoxville-1	1

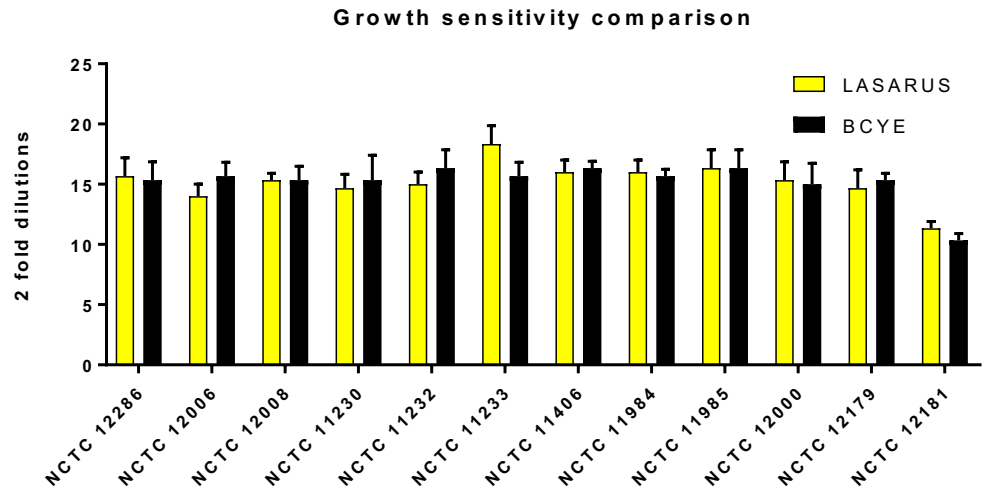
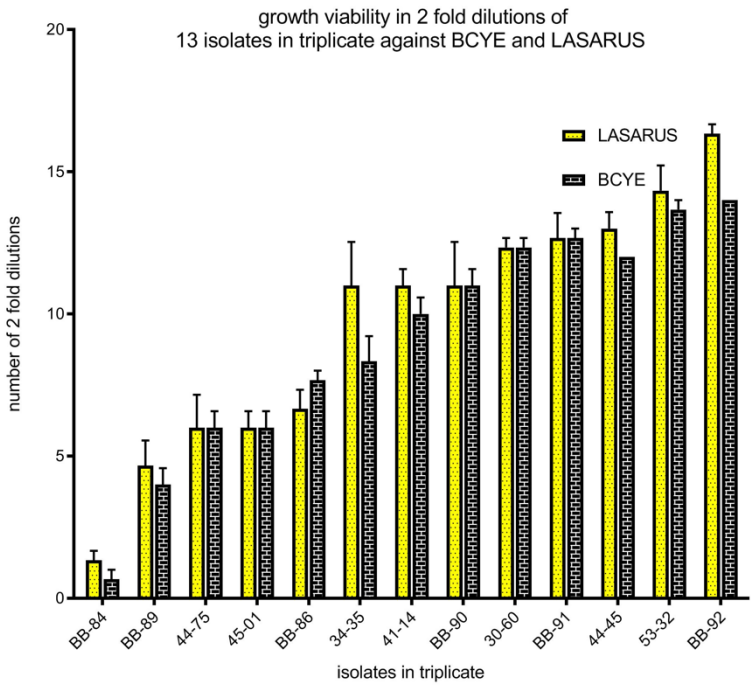


Figure 4.1 A Growth comparison of BCYE-α and LASARUS in 12 isolates

An analysis in triplicate between BCYE-α showing similar growth patterns (P=>0.99) (Discovery determined using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q= 1%. Each row was analyzed individually, without assuming a consistent SD.

This comparison of 12 NCTC strains showed that prototype strains that may have been conditioned to BCYE-α grow similarly on LASARUS (p=>0.99, paired T-test).

A repeated experiment using low passage clinical isolates Figure 4.2 and Figure 4.3 below confirmed LASARUS was as good as BCYE-α for *Legionella* growth. Figure 4.2 shows a comparison of BCYE-α and LASARUS growth variability in 2-fold dilutions of 13 isolates grown in triplicate. A 2-way ANOVA showed that these were statistically not different (p=0.69). Figure 4.3 showed that Cumulative data comparison which suggests slightly better growth seen for LASARUS compared to that seen than with BCYE-α. However, an unpaired T-test showed that this difference was not statistically significant (p=0.47).



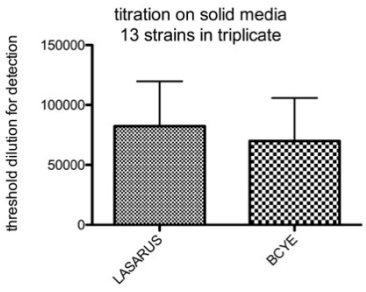


Figure 4.2 A Comparison of BCYE-α and LASARUS growth variability
Growth variability on 2-fold dilutions of low passage clinical isolates

Figure 4.3 B Cumulative data comparison (BCYE-α vs LASARUS)
Cumulative data for the above shown growth variability

Figure 4.4 and Figure 4.5 illustrates examples of inoculated plates. Note that the picture of LASARUS was obtained through the bottom of the plate, while BCYE-α required removal of the plate lid (due to condensation) for imaging, further highlighting the safety advantages of a clear media such as LASARUS.

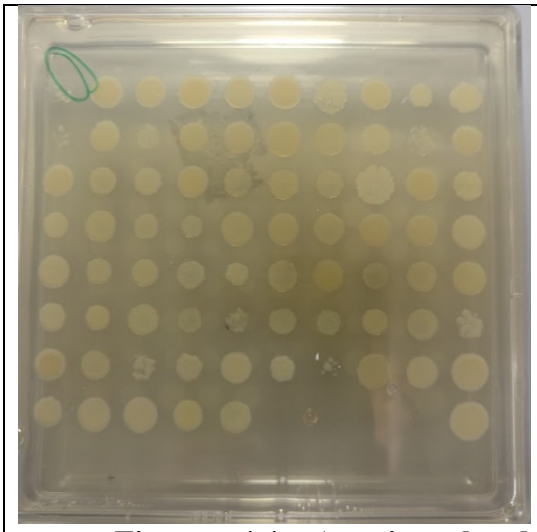


Figure 4.4 An inoculated LASARUS plate

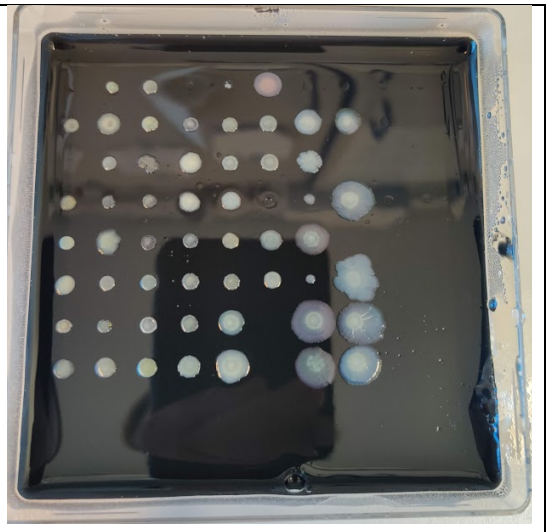


Figure 4.5 An inoculated BCYE-α plate

Figure 4.4 and Figure 4.5 show a LASARUS and a BCYE-α plate inoculated with *Legionella* and incubated for five days at 37ºC.

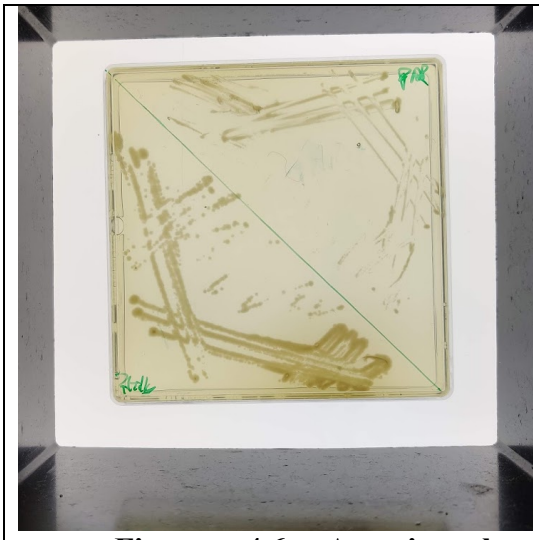


Figure 4.6 An inoculated LASARUS plate

Figure 4.7 An inoculated BCYE-α plate

Figure 4.6 and Figure 4.7 show a LASARUS and a BCYE-α plate inoculated with *Legionella pneumophila* Paris and Philadelphia and incubated for five days at 37ºC.

There are no internationally agreed MIC ranges for any antibiotic and *Legionella* spp. Therefore, prior to high throughput *Legionella* AST was performed I compared LASARUS to the traditional gold standard for MIC agar dilution, Mueller Hinton (MH). As *Legionella* spp. wouldn't grow on MH agar, the growth of ATCC strains

Escherichia coli 25922, *Staphylococcus aureus* 29213, *Pseudomonas aeruginosa* 27853 and a bla_{oxa+} *Klebsiella pneumoniae* was compared. These isolates were tested on a range of antibiotics with internationally defined MICs with MH. The results showed a 2-4-fold increase in MIC when grown on LASARUS compared to MH, as can be seen in the Table 24. The difference is likely due to the greater nutrient content of LASARUS relative to the sparse content of MH. Anecdotal evidence supporting this theory comes from the observation that after 24 hours incubation the colony size was roughly twice the size on LASARUS compared to MH for all bacteria in Table 24.

Table 24 AST comparison of Mueller Hinton agar (MH) with LASARUS (LAS)

LASARUS showed differences of between 1-4times that of MH. All MICs in (µg/mL)

		S. aureus		*E. coli*		*P. aeruginosa*		*K. pneumoniae*	
Antibiotics	Ranges tested	MH	LAS	MH	LAS	MH	LAS	MH	LAS
Ampicillin	0.5-8	4	2	>8	>8	>8	>8	>8	>8
Gentamycin	0.125-16	0.125	0.5	1	0.5	1	4	0.25	1
Ciprofloxacin	0.004-2	1	0.5	0.016	0.03	0.125	0.5	0.5	1
Levofloxacin	0.008-2	0.25	0.25	0.03	0.03	0.5	2	2	>2
Azithromycin	0.5-2	2	2	1	>2	>2	>2	>2	>2
Tetracycline	0.125-1	>1	0.5	>1	>1	>1	>1	>1	>1
Rifampicin	0.004-0.03	0.016	0.016	>0.03	>0.03	>0.03	>0.03	>0.03	>0.03
Chloramphenicol	2-16	4	4	2	4	>16	>16	>16	>16

While this raised concerns that LASARUS may give elevated MICs compared BCYE-α for *Legionella* spp., the appropriate comparison is of LASARUS relative to the gold standard for *Legionella* MIC testing; BMD, and this is systematically investigated in the next chapter (Portal et al. 2021a).

4.4.4 The importance of pH on Legionella growth.

In negotiations and batch testing that took place as part of attempted commercialisation of LASARUS, it was demonstrated that BCYE-α performance was linked to pH (collaborative data covered by NDA). The importance of pH for growth of certain *Legionella* spp. has been previously published (Fliermans et al. 1981). However, testing the pH of a solid was not readily achievable with the available equipment in Cardiff, but utilising equipment at Public Health England I tested six randomly selected LASARUS plates using a Mettler Toledo™ Combination Electrode for Surface Measurements pH reader that consistently gave an average pH of 7.2. This was elevated compared to commercial BCYE-α plates which had a pH of 6.9, the published target pH (+/-0.2) according to https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Datasheet/1/86558dat.pdf.

While routine pH measurement of agar plates could not be performed, the pH of the ACES stock buffer component could be increased or decreased by 0.2 pH units prior to sterile filter sterilisation. As outlined above, the pH was adjusted with either 0.1M or H_2SO_4 or 0.1M KOH to avoid issues with sodium. Figure 4.8 shows the relative growth for representative serogroups from Table 3.5, assessed by inoculation of a 2-fold dilution series on LASARUS plates with pH of 7.2 (original), 6.9 or 6.7 relative to BCYE-α.

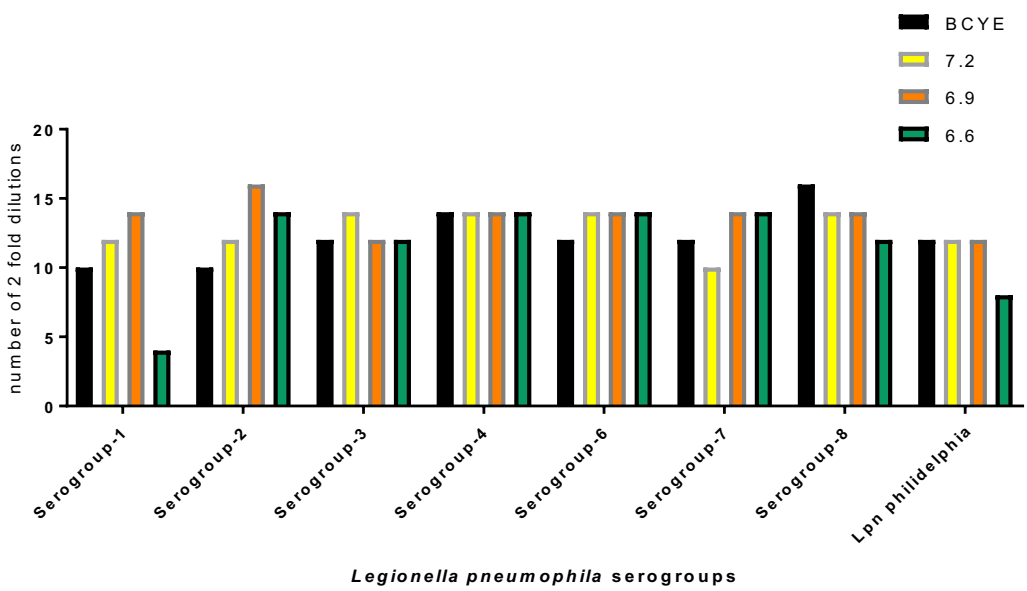


Figure 4.8 Effect of varying pH on *Legionella* typed strains

Comparison of *Legionella pneumophila* strains growth on LASARUS plates at differing pH 6.6, 6.9 and 7.2 as well as a control BCYE-α plate.

Legionella pneumophila prototype strains and NCTC were not significantly affected by pH with the exception of two separate Lp Sg 1 strains (including Philadelphia strain) that had a reduced titre for the pH 6.6 test plate. Furthermore, two strains showed improved growth on LASARUS with a pH 6.9. This systematic investigation was then extended to six non-*pneumophila Legionella* spp. Strains (Figure 3.5).

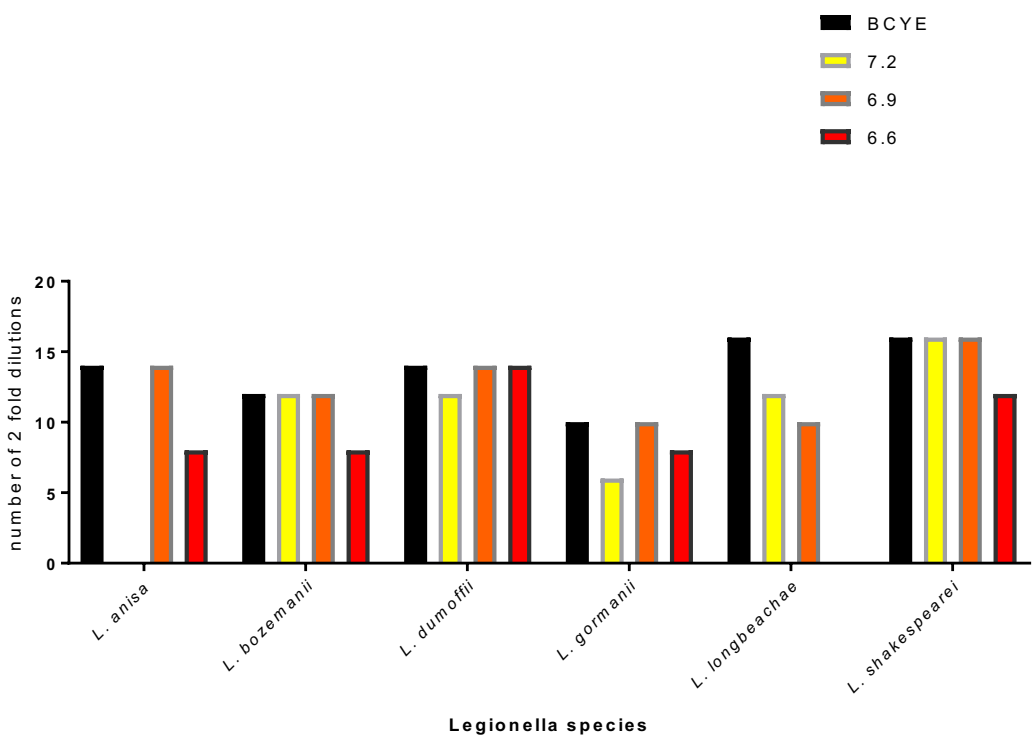


Figure 4.9 Effect of varying pH on non-*pneumophila Legionella* strains
Comparison of non-pneumophila *Legionella* spp. strains titrated on LASARUS plates at pH 6.6, 6.9 and 7.2 as well as a control BCYE-α plate. *Legionella londinensis* failed to grow on any LASARUS media.

In contrast to *Legionella pneumophila* species, non-pneumophila species were more affected by pH modification: only *L. bozemanii* and *L. shakespearei* grew equivalently on LASARUS 7.2 compared to BCYE-α. With the exception of *L. longbeachae*, LASARUS with a pH 6.9 consistently gave the best performance. *L. anisa* and *L. gormanii* were found to be the most sensitive to small alterations to pH.

4.4.5 The importance of cations on Legionella growth

Following on from the CDC document indicating pH must be adjusted with KOH, not NaOH. I designed an MIC-style experiment examining the concentration at which KCl, NaCl, $CaCl_2$ and $MgCl_2$ inhibited the growth of a panel of 72 assorted *Legionella*

pneumophila. Shown in Figure 4.10 made by performing an agar dilution experiments where each point represents the percentage of isolates which are growing at each concentration point. Increasing salt concentration decreases number of isolates viable. NaCl (increments of 20mM from 60 to 160mM), KCl, (increments of 30mM from 140 to 290mM), MgCl2, (increments of 4mM from 12 to 36mM), CaCl2 (1.17, 2.34 4.68, 9.37 and 18.75mM). These experiments were performed as dilution on LASARUS agar as per other MIC experiments see methods MIC General 3.4.

Figure 4.10 shows that KCl did not significantly inhibit *Legionella* growth as compared to NaCl which at (homeostatic concentration (0.9%; 150mM) resulted in a loss of 50% of the strains as can be seen in Table 25. This means that protocols which routinely require the MacFarland standard of *Legionella* spp. to be diluted in saline prior to plate inoculation, must be changed to ensure that the saline is not interfering in cellular viability. As such all future experiments were carried out in MGW.
There was significant reduction in strain viability with $MgCl_2$ (complete inhibition of all strains at 37.5 mM) and $CaCl_2$ (complete inhibition at 9.4 mM), that shows the presence of these cations must be avoided in any *Legionella* growth medium and suggests that chelation of Ca^{2+} in particular would benefit *Legionella* spp. growth. Table 25 shows the MIC_{50} and MIC_{90} of the above tested 72 isolates highlights the potency of $MgCl_2$, $CaCl_2$.

This work was done with the assistance of Rupert Kelly an undergraduate student I supervised during his dissertation research project.

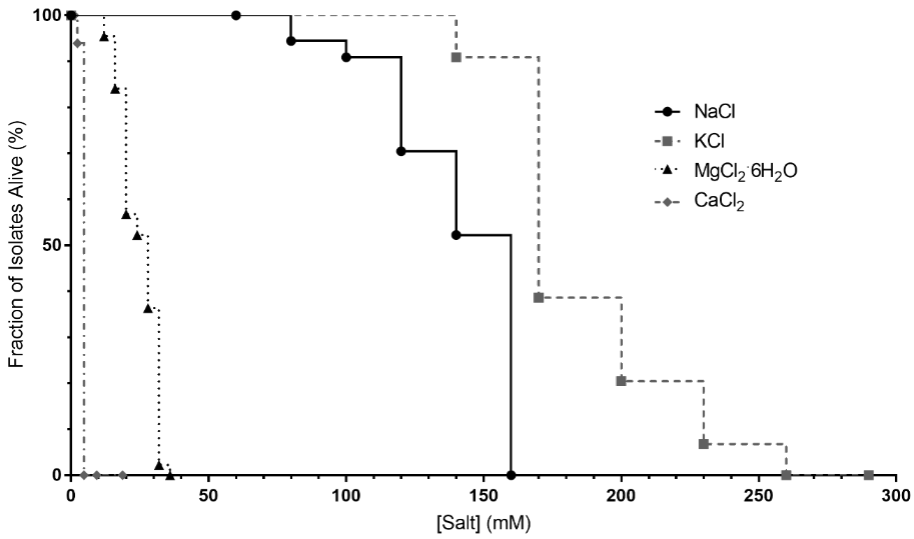


Figure 4.10 A Kaplan Meier graph showing the effect of increasing salt concentrations on *Legionella pneumophila* viability.

Table 25 MIC_{50} and MIC_{90} in mM for the 72 isolates tested against these salts

Cation	MIC_{50} (mM)	MIC_{90} (mM)
NaCl	140	160
KCl	170	230
$MgCl_2$	28	32
$CaCl_2$	4.68	4.68

4.4.6 Effects of ion chelation

The presence of calcium and magnesium adversely impacted on *Legionella* spp. growth on LASARUS as shown in Table 25 and in Figure 4.10. As serum and yeast extract contain cations, a cation chelator was used to offset the effects of the calcium and magnesium which significantly reduced *Legionella* growth as shown in Table 25 and in Figure 4.10 in an attempt to maximise the growth capacity of LASARUS for *Legionella* spp. Compound E is a chelating agent.

The MIC of Compound E for a panel of 38 *Legionella* "non" *pneumophila* species as well as 16 NCTC *Legionella pneumophila* prototype strains was between 962 to >3,848µg/mL, indicating negligible inhibition, but more importantly of 18 the *Legionella* spp. strains that failed to grow on the Compound E -free control plates; ten actively grew on the agar Compound E dilution gradient plates in the same experiment (Table 26).

This table shows the extremely high concentrations required for inhibition of *Legionella* growth. As well as the ability for Compound E to induce growth of strains that had failed to grow on the Compound E -free control LASARUS plate these have been highlighted in yellow.

This demonstrates the potential for Compound E to improve LASARUS.

Table 26 Growth effect and MIC of Compound E on *Legionella* species panel with pneumophila prototype strains

Final MIC concentrations for Compound E are shown in µg/mL.

Legionella Species	Growth on		
	Compound E free plate	any Compound E plate	MIC to Compound E
L. adelaidensis	No	No	No growth
L. anisa	No	Yes	962
L. beliardensis	Yes	Yes	>3848
L. birminghamensis	Yes	Yes	3848
L. bozemanii sg1	Yes	Yes	1924
L. bozemanii sg2	Yes	Yes	1924
L. brunensis	No	Yes	3848
L. busanensis	Yes	Yes	3848
L. cherrii	No	Yes	962
L. cincinnatiensis	Yes	Yes	1924
L. donaldsonii	Yes	Yes	3848
L. dresdenensis	Yes	Yes	1924
L. dumoffii	Yes	Yes	3848
L. erythra	Yes	Yes	3848
L. fairfieldensis	No	Yes	1924
L. feeleii	Yes	Yes	3848
L. feeleii	Yes	Yes	>3848
L. sp.	Yes	Yes	1924

L. gratiana	Yes	Yes	3848
L. hackeliae	Yes	Yes	1924
L. hackeliae	Yes	Yes	>3848
L. israelensis	No	Yes	1924
L. jamestownensis	Yes	Yes	3848
L. lansingensis	No	Yes	>3848
L. londiniensis	Yes	Yes	3848
L. longbeachae sg1	Yes	Yes	1924
L. maceachernii	Yes	Yes	3848
L. micdadei tatlock	Yes	Yes	3848
L. micdadei heba	Yes	Yes	1924
L. nagasakiensis	Yes	Yes	1924
L. oakridgensis	Yes	Yes	1924
L. parisiensis	No	Yes	1924
L. quinliviannii sg1	Yes	Yes	3848
L. rubrilucens	No	Yes	1924
L. sainthelensi sg1	Yes	Yes	1924
L. sainthelensi sg2	Yes	Yes	1924
L. shakespearei	Yes	Yes	3848
L. spiritensis sg2	Yes	Yes	3848
L. tucsonensis	No	Yes	1924
L. wadsworthii	No	Yes	962
L. pneumophila NCTC12286	Yes	Yes	>3848
L. pneumophila NTCT11404	Yes	Yes	>3848
L. pneumophila NCTC12007	Yes	Yes	>3848
L. pneumophila NTCT12008	Yes	Yes	>3848
L. pneumophila NTCT12024	Yes	Yes	>3848
L. pneumophila NTCT11230	Yes	Yes	>3848
L. pneumophila NTCT11232	Yes	Yes	>3848
L. pneumophila NTCT11233	Yes	Yes	3848
L. pneumophila NTCT11406	Yes	Yes	>3848
L. pneumophila NCTC11984	Yes	Yes	>3848
L. pneumophila NTCT11985	Yes	Yes	>3848
L. pneumophila NCTC12000	Yes	Yes	>3848
L. pneumophila NTCT12179	Yes	Yes	>3848
L. pneumophila NCTC12180	Yes	Yes	>3848
L. pneumophila NCTC12181	Yes	Yes	3848
L. pneumophila NCTC12174	Yes	Yes	>3848

Compound E is not traditionally considered a growth promotor or enhancer, but its primary effect in the chelation of metal ions, specifically calcium, appears to have a

positive effect on *Legionella* growth. For all experiments that follow a concentration of 471µg/mL was chosen to maximise growth promotion but minimise the risk of inhibition.

Figure 4.11 shows the effect of Compound E on the growth of 70 isolates (29 *Legionella pneumophila* strains including serogroup prototype strains from Table 23 and 41 other *Legionella* spp.) at pH 7.2 (in triplicate). Statistical analysis of all isolates together showed a significant growth of isolates comparing LASARUS with and without added Compound E ($p=0.005$).

However, this difference was more pronounced when removing the *L. pneumophila* strains and reanalysing the effect of Compound E (Figure 4.12; $p<0.0001$). During attempts to negotiate licencing of LASARUS manufacturing with commercial media suppliers, it was indicated that *L. anisa* was universally accepted in the manufacturing sector to be the most problematic to culture. Figure 4.13 shows that *L. anisa* would only grow on LASARUS at pH 7.2 with the addition of Compound E.

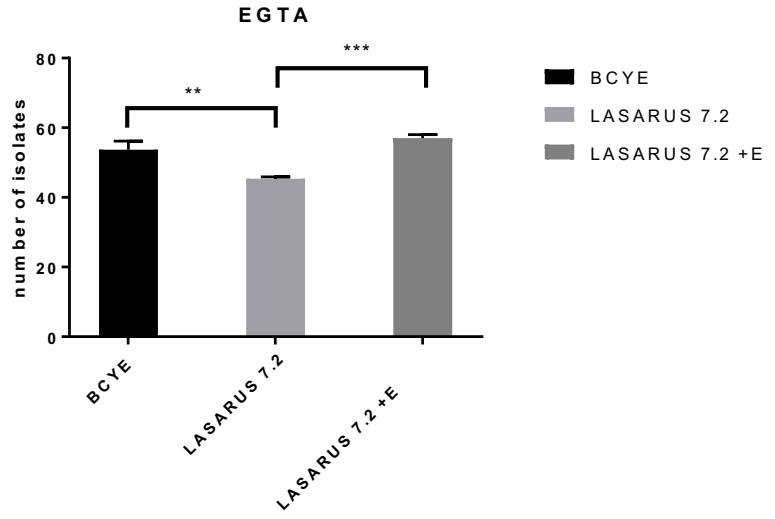


Figure 4.11 Effect on growth of *Legionella* with the addition of Compound E to LASARUS

Effect of Compound E on *Legionella pneumophila* strains (including serogroup prototype strains from Table 23). The graph shows the number of isolates with recorded growth on BCYE-α, LASARUS and LASARUS supplemented with Compound E. A one-way ANOVA with *post hoc* analysis with Tukeys (correcting for multiple comparisons) showed statistical significance with **=p<0.01 ***=p<0.001.

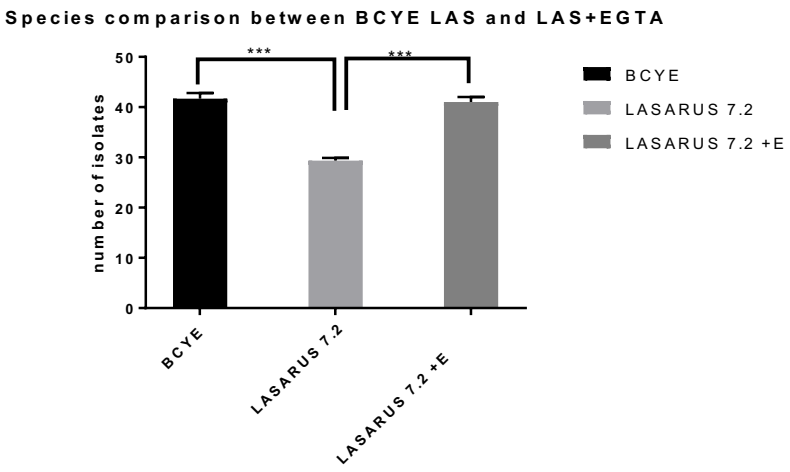


Figure 4.12 Effect on growth of non pneumophila *Legionella* with the addition of Compound E to LASARUS

Reanalysis of data in Figure 4.11 after excluding the *L. pneumophila* strains. This illustrates the number of strains showing growth on BCYE-α, LASARUS and LASARUS supplemented with Compound E in triplicate. A one-way ANOVA with *post hoc* analysis with Tukeys (correcting for multiple comparisons) showed statistical significance with ***=p<0.001.

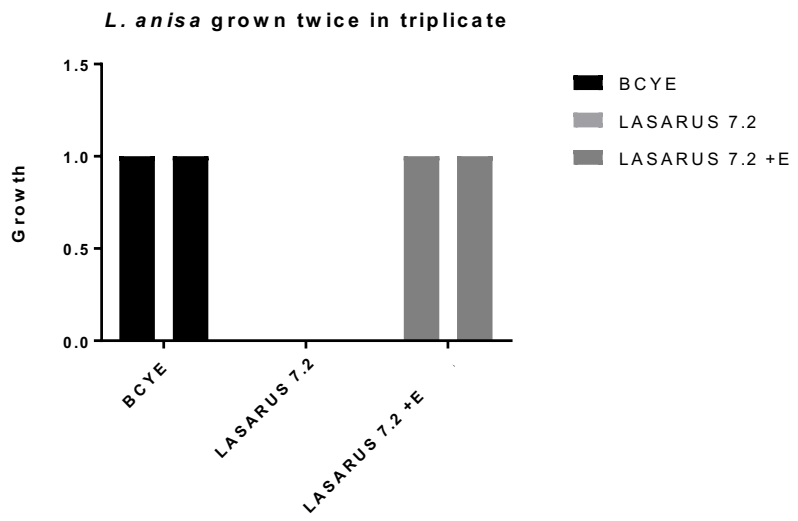


Figure 4.13 Effect of Compound E on growth of *Legionella anisa*

Effect of Compound E on growth of *Legionella anisa,* repeated twice in triplicate. No growth was observed for LASARUS at pH 7.2 unless supplemented with Compound E consistently.

4.4.7 Fully optimised LASARUS formulation

pH and removal of cations each contributed significantly to improve *Legionella* spp. growth on LASARUS. In this section, a systematic investigation of combining these two variables for optimised growth is detailed. The pH concentrations 6.6, 6.9 and 7.2 were all compared with and without Compound E at 471µg/mL in triplicate and at different CFU concentrations. Sixty isolates including representatives of all *Legionella* spp as well as the *Legionella pneumophila* serogroup panel (Table 23) were used for evaluation. The original experimental design incorporated four different concentrations of bacterial inoculi, however as there was no noticeable difference between the tests, data is only presented (Figure 3.11) for the routine concentration of 10% of a 0.5Mcfarland standard (which is the concentration set for multipoint inoculation, Kirby-Bauer disk diffusion and E-tests in antimicrobial susceptibility testing, EUCAST, disk diffusion method, Version 9.0).

Figure 4.14 shows a comparison of *Legionella* spp. growth on LASARUS formulated at pH of 7.2, 6.9, and 6.6 with and without Compound E; all compared to growth on BCYE-α. Number of isolates successfully grown (out of a total of 60) are shown. The error bar showing the SD and the bar representing the mean of the three replicates. This has shown without separation by species, statistical analysis (one-way ANOVA with *post-hoc* Tukey test, corrected for multiple comparisons) is shown in Table 27.

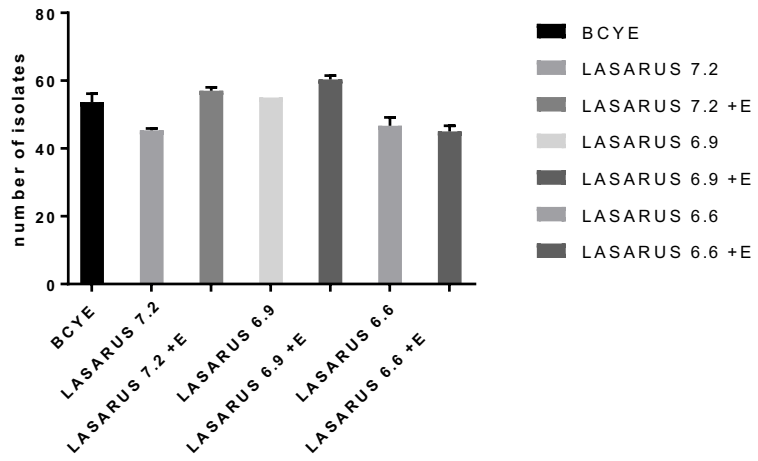


Figure 4.14 *Legionella* spp. growth on LASARUS pH6.6/6.9/7.2 +/- Compound E

Table 27 Statistical analysis comparing pH and Compound E on *Legionella* growth.

TEST	Significance	P Value
BCYE-α vs. LASARUS 7.2	***	0.0003
BCYE-α vs. LASARUS 7.2 + Compound E	NS	0.2227
BCYE-α vs. LASARUS 6.9	NS	0.9435
BCYE-α vs. LASARUS 6.9 + Compound E	**	0.0026
LASARUS 7.2 vs. LASARUS 7.2 + Compound E	****	<0.0001
LASARUS 7.2 vs. LASARUS 6.9	****	<0.0001
LASARUS 7.2 vs. LASARUS 6.9 + Compound E	****	<0.0001
LASARUS 7.2 + Compound E vs. LASARUS 6.9	NS	0.7333
LASARUS 7.2 + Compound E vs. LASARUS 6.9 + Compound E	NS	0.2227
LASARUS 6.9 vs. LASARUS 6.9 + Compound E	*	0.0162
One-way ANOVA with *post-hoc* Tukey test, corrected for multiple comparisons. NS= not significant.		

The key finding from Table 27 is that changing the pH to 6.9 as well as the addition of Compound E are cumulative and has the capacity to sustain a range of *Legionella* species

and is improved relative to both the original LASARUS formulation (p=<0.0001) and is also a significant improvement on BCYE-α (p=0.0026).

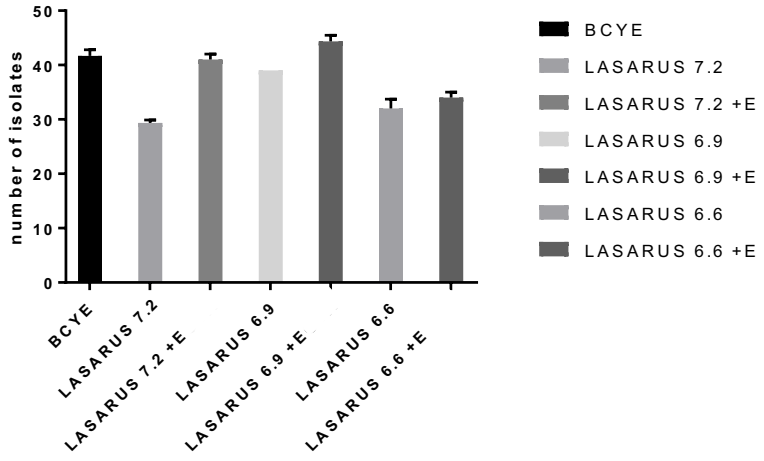


Figure 4.15 Non-*pneumophila Legionella* spp. growth on LASARUS pH6.6/6.9/7.2 +/- Compound E

Figure 4.15 which shows a comparison of 44 *Legionella* spp. excluding *L. pneumophila* for the effect of LASARUS formulated at pH of 7.2, 6.9, and 6.6 with or without Compound E; all compared to growth on BCYE-α. Mean and standard deviation are shown for triplicate results. Statistical analysis (one-way ANOVA with post-hoc Tukey test, corrected for multiple comparisons) is shown in Table 28.

Table 28 Statistical analysis comparing the effects of pH and Compound E on non *pneumophila Legionella* spp. growth

Comparison	Significance	P Value
BCYE-α vs. LASARUS 7.2	****	<0.0001
BCYE-α vs. LASARUS 7.2 + Compound E	NS	0.9850
BCYE-α vs. LASARUS 6.9	NS	0.0938
BCYE-α vs. LASARUS 6.9 + Compound E	NS	0.0938
LASARUS 7.2 vs. LASARUS 7.2 + Compound E	****	<0.0001
LASARUS 7.2 vs. LASARUS 6.9	****	<0.0001
LASARUS 7.2 vs. LASARUS 6.9 + Compound E	****	<0.0001
LASARUS 7.2 +Compound E vs. LASARUS 6.9	NS	0.3121
LASARUS 7.2 +Compound E vs. LASARUS 6.9 + Compound E	*	0.0241
LASARUS 6.9 vs. LASARUS 6.9 + Compound E	***	0.0004

One-way ANOVA with *post-hoc* Tukey test, corrected for multiple comparisons. NS= not significant.

The greatest effect of pH and cation chelation was noted when analysing growth of non-*pneumophila Legionella* species as can be seen in Figure 4.15 and statistically proven in in Table 28. Whilst the trend for LASARUS 6.9+ Compound E appears to produce better growth than BCYE-α, this was not statistically significant (p=0.0938). Some *Legionella* species are much more sensitive and grow significantly worse as compared to *Legionella pneumophila*. To demonstrate the effects of pH and Compound E on the most sensitive *Legionella* spp. and show a binary output (growth=1 no growth=0) for experiments carried out in triplicate.

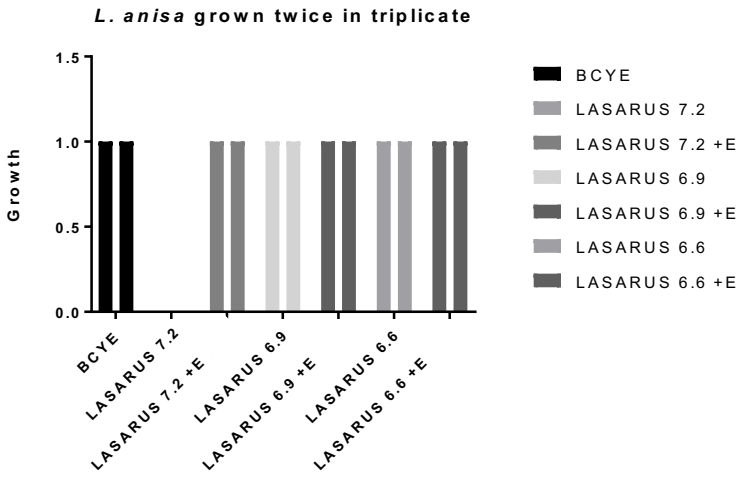


Figure 4.16 LASARUS pH and Compound E for *Legionella anisa* relative to BCYE-α growth

Effect of LASARUS pH and Compound E for *Legionella anisa* relative to BCYE-α growth. *L. anisa* grew on all LASARUS agars bar LASARUS 7.2 without Compound E. Results for two biological replicates of the comparison (carried out in triplicate) were performed to ensure veracity of the findings.

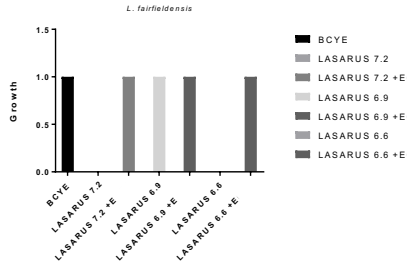


Figure 4.17 LASARUS pH and Compound E for *Legionella farfieldensis* relative to BCYE-α growth.

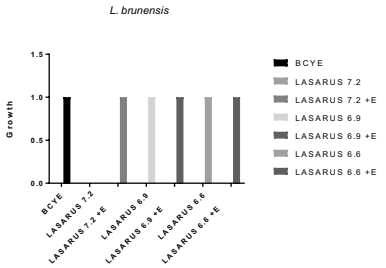


Figure 4.18 LASARUS pH and Compound E for *Legionella brunensis* relative to BCYE-α growth

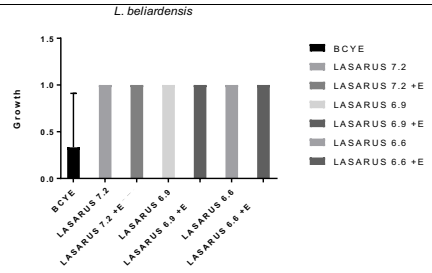


Figure 4.19 LASARUS pH and Compound E for *Legionella belardensis* relative to BCYE-α growth

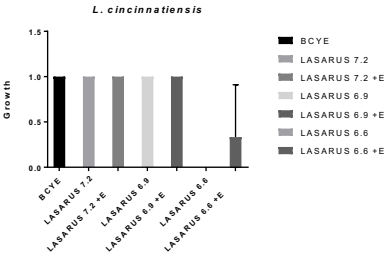


Figure 4.20 LASARUS pH and Compound E for *Legionella cincinnatiensis* relative to BCYE-α growth

The effect of modifying pH and Compound E for *Legionella farfieldensis (*Figure 4.17*)* which grew on all LASARUS agars bar LASARUS pH7.2 and pH6.6 both without Compound E. *Legionella brunensis (*Figure 4.18*)* grew on all LASARUS agars apart from LASARUS pH7.2 with and without Compound E. Similarly with *L. anisa* and *Legionella belardensis (*Figure 4.19*)* grew on all LASARUS agars equivalently, but less well on BCYE-α (only one third of the inoculated plates showed growth). *Legionella cincinnatiensis* grew on all LASARUS agars except those at pH 6.6 and it grew poorly on pH6.6+Compound E.

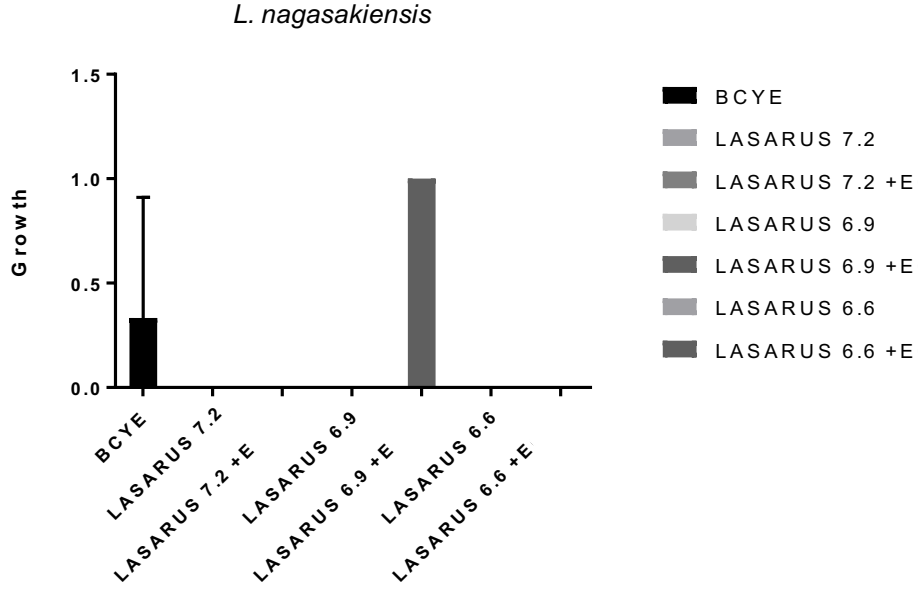


Figure 4.21 LASARUS pH and Compound E for *Legionella nagasakiensis* relative to BCYE-α growth

Effect of LASARUS pH and Compound E for *Legionella nagasakiensis* relative to BCYE-α growth. *L. nagasakiensis* only grew on LASARUS at 6.9 with Compound E, which performed better than BCYE-α (only 1/3 plates grew).

Legionella nagasakiensis is one of the strains along with *L. anisa* that was consistently the most difficult to culture. For both of these only a third of plates showed growth on BCYE-α (keeping in mind this has come from a growing culture on a BCYE-α plate), and the species only grew on LASARUS 6.9+Compound E, further highlighting its superiority to the original LASARUS media as well as to BCYE-α.

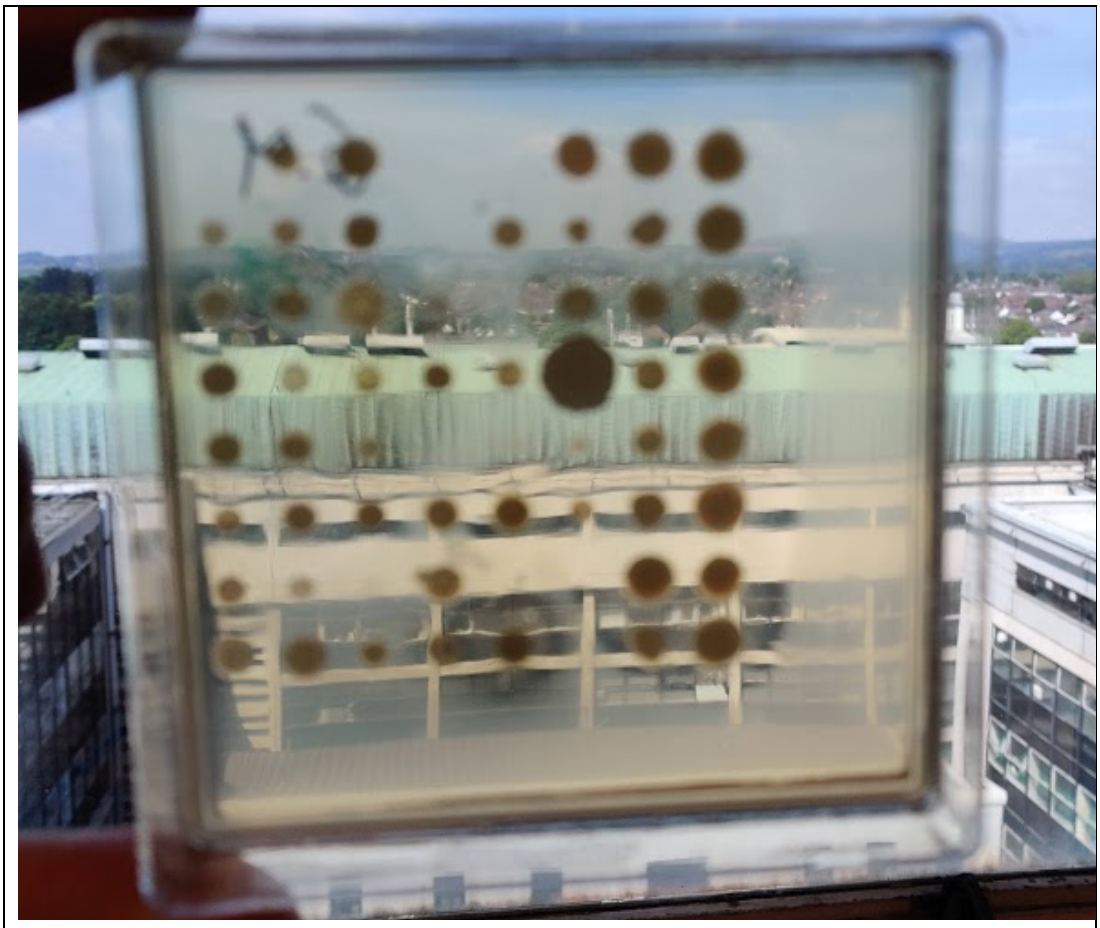


Figure 4.22 An interesting side effect
Picture of *Legionella* species on a Lasarus6.9+Compound E plate, highlighting the regions of increased transparency around the colonies. The importance of this is unknown.

Figure 4.22 highlights an interesting effect seen when growing *Legionella* spp. on LASARUS pH6.9+Compound E. These plates are translucent when poured and remain so with storage; however, growth of *Legionella* create a zone of greater transparency. Whether this is due to greater metabolism of the nutrients in the surrounding area or excretion of enzymes that breakdown the complex components of the media adjacent to the colonies is unknown. However, this effect may be of future utility in diagnostics. This could be an anti-serum factor or a lipase reacting with the sheep serum in the agar it is particularly interesting to note that this does not happen

without Compound E in the plate, it is not consistent with all *Legionella* isolates and on a BCYE-α plate this effect would be undetectable.

4.5 Discussion

4.5.1 Alternative media

As summarised in sections 4.2 several researchers have tried and, in some cases, partially succeeded in creating media alternative to BCYE-α for growing *Legionella* species. I have highlighted the unsuitability of some in sections 4.2.4 based on safety or limitations to mass production and have been unable to reproduce or show sufficient merit of those that I repeated in section 4.3.1. The most successful potential alternative was Washed Buffered Yeast Extract (WBYE) from Rogers *et al* in 1992, which had the potential to remove toxins from the agar prior to autoclaving. However, WBYE agar was tested by Nielsen, *et al.* who found that growth was totally inhibited in 20% of isolates using this medium (Nielsen et al. 2000). My attempts to replicate the original studies were not successful, as I failed to form a sufficiently solid media on multiple occasions. In the formulation of LASARUS several buffering alternatives were evaluated. Tris and MOPS failed to support growth of *Legionella* which was unexpected as they possess similar buffering range capacities to ACES and MOPS had been reported previously to function as substitutes for ACES (Armon and Payment 1990). This was disappointing as MOPS is significantly cheaper than ACES buffer. (£363/Kg compared to £1074.60/Kg), and more readily available.

4.5.2 Safety cost and ease of manufacture

Safety was a key factor in the creation of the LASARUS media the formulation was similar to BCYE-α, and using LASARUS for investigations with *Legionella* spp. is easier and safer then on BCYE-α, as colonies can be observed through the bottom of

the plate without removing the lid and exposing the researcher to a pulmonary pathogen. Condensation accumulates on the lid of a BCYE-α plate, observation of growth is best handled using a bacterial containment laminar flow hood.

From a cost to manufacture perspective, LASARUS is comparable to BCYE-α, where the most expensive component is the ACES buffer, due to the requirement for L-cysteine and alpha-ketoglutarate addition, the production of BCYE-α has always been a 2-step manufacturing process, one part autoclaved, and one part filter sterilised, LASARUS is no different and the production times are the same.

4.5.3 Further development of LASARUS

pH is known to affect *Legionella pneumophila* and it has been shown that when free living (*in vitro*) *Legionella pneumophila* survives and replicates in a pH range of between 6.0 and 8.0 (Wadowsky et al. 1985; Ohno et al. 2003). Katz and Hammel in 1987 found a 2-log decrease in live cells after a month-long suspension in water ranging from a pH of 4.0 to 7.0, however when the pH was increased to 8.0 they found a 6-log decrease in viable cells (Katz and Hammel 1987). In addition, the published pH in the formulation of commercial *Legionella* growing medium is always between 6.6-6.9.

Therefore, one would predict the optimal pH range for *Legionella pneumophila* growth is between 6.0-8.0 and *Legionella pneumophila* was less sensitive to a pH range than other species.

Non-*pneumophila Legionella* species are less well characterised and cause a small fraction (less than 10% worldwide) (Muder and Yu 2002) of disease; therefore, they were not a consideration in the early development of LASARUS, however their

growth was a consideration of the later stages of LASARUS development and I have shown how LASARUS can now grow these species.

The optimised pH of LASARUS was pH 6.9 with Compound E at a concentration of 471µg/mL. The next phase in the evolution of LASARUS will be to reduce manufacturing cost and increase efficiency and ease of production for commercial production. After which an investigation into the feasibility of meeting HSE requirements for comparative cfu/L from environmental isolation will be attempted.

4.6 Conclusion

This chapter has focussed on the production of a novel media for growing *Legionella* spp. This media was needed as performing MIC analysis using any of the other available methods (BMD and BCYE-α agar dilution) are heavily time consuming or highly variable. This involved evaluating pre-existing media, then systematically eliminating them based on failure to meet key criteria i.e not contain chelating compounds, activated charcoal, comparable to BCYE-α, able to grow all *Legionella* spp. have a reasonable cost relative to BCYE-α, be easy and safe to manufacture relative to BCYE-α production and ideally be transparent/translucent.

Pre-existing media were discounted and formulating a novel media was chosen. LASARUS was formulated, improved and is patent protected for UKHSA and Cardiff University by Mathys & Squire LLP. Patent number: WO2020039213A1.

The next chapter systematically compares LASARUS, BMD and BCYE-α (agar and E-test) to determine MIC for *Legionella* spp.

5 Results Chapter 2 Concordance

5.1 Introduction

This chapter primarily focuses on the comparison of MICs determined for a panel of 50 *Legionella* strains consisting of 35 Welsh clinical and environmental isolates, combined with five strains from a previously published BMD (Wilson et al. 2018) study and ten NCTC strains of different serogroup as no reference strains had been previously agreed upon; for BMD and BCYE-α (antimicrobial-gradient strips and dilution of antimicrobials in agar) relative to a new novel commercially available charcoal-free *Legionella* Antimicrobial Susceptibility And Resistance Universal Screening medium (LASARUS). This chapter provides a basis for future studies to evaluate, in a validated manor, resistance value and range points to enable internationally agreed and defined resistance breakpoints for Legionellaceae.

This chapter has been published in the Journal of Antimicrobial Chemotherapy (Portal et al. 2021a)

5.2 Results

5.2.1 Reference cohort

A previously unused fifty *L. pneumophila* strains were included in the study previously archived at Public Health England (50 *Legionella pneumophila*): 22 clinical strains from Wales, 13 environmental strains from Wales, five previously reported strains from England and 10 NCTC reference strains seen in Table 29. Figure 5.1 is a schematic of the workflow used for the main experiments in this chapter. Which compares BCYE agar dilution, broth microdilution (BMD) in *Legionella* Growth Medium (LGM), and agar dilution in LASARUS.

Table 29 *Legionella* typed strains in the reference panel

Legionella NCTC	Serogroup
NCTC-11192	1
NCTC-12286	1
NCTC-12006	1
NCTC-12024	1
NCTC-11232	3
NCTC-11984	7
NCTC-11985	8
NCTC-12180	12
NCTC-12181	13
NCTC-12174	14

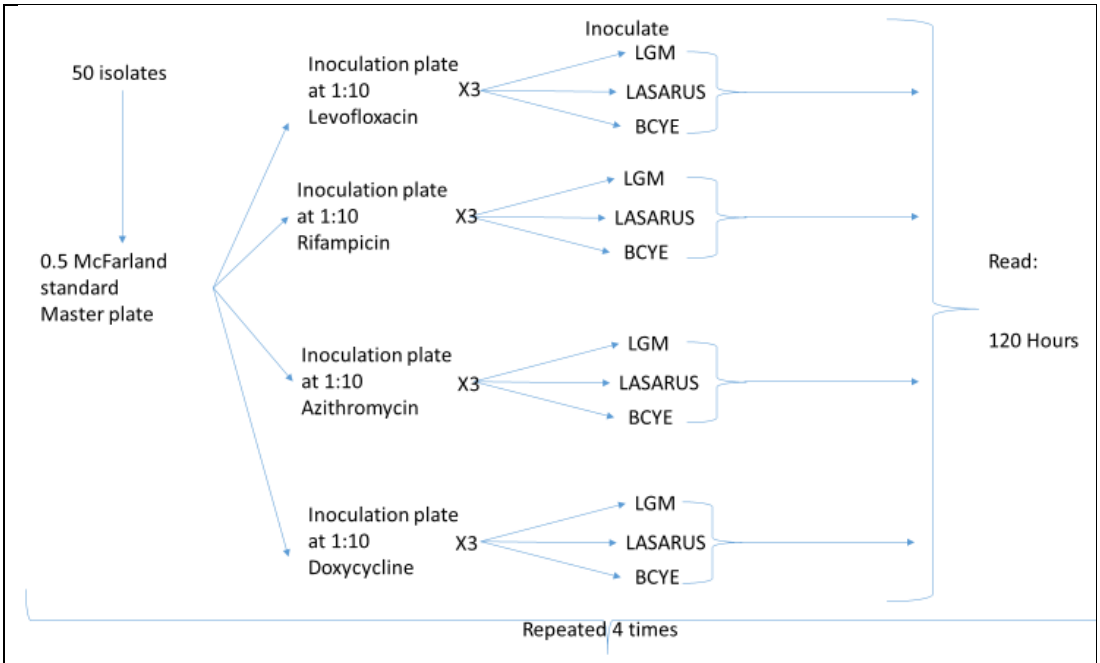


Figure 5.1 A schematic of the primary methodology for this experiment

5.2.2 Comparison of four methodologies for MIC determination: (BMD, gradient strip (BCYE-α), solid agar (LASARUS, BCYE-α).

There was a close concordance between liquid (BYE-α) medium and LASARUS agar for the determination of minimum inhibitory concentrations for rifampicin (Figure 5.2), levofloxacin (Figure 5.3) and azithromycin (Figure 5.5). The thresholds of inhibition were found to be moderately elevated for gradient strip tests on BCYE-α and remarkably elevated in BCYE-α agar dilution.

MIC_{50} and MIC_{90} for rifampicin determined on LASARUS or by BMD with BYE-α gave values of 0.004µg/mL and of 0.008µg/mL, respectively. Values for gradient strip tests gave MIC_{50} of 0.032µg/mL and MIC_{90} of 0.032µg/mL, while for BCYE-α agar dilution values were elevated 16-fold to MIC_{50}=0.06µg/mL and MIC_{90}=0.128µg/mL (Figure 5.2).

Similarly, levofloxacin MIC values determined on LASARUS and by BMD with BYE-α were identical with both having MIC_{50}=0.03µg/mL and MIC_{90}=0.03µg/mL, compared to 32-fold higher MIC_{50}=1µg/mL and MIC_{90}=1µg/mL for BCYE-α dilution, while gradient strip tests gave intermediate but two fold increase in elevated MIC_{50}=0.064µg/mL and four fold on the MIC_{90}=0.128µg/mL Figure 5.3.

Relative to other tested antimicrobials, substantially higher concentrations of doxycycline were required to inhibit the growth of the 50 isolates of *L. pneumophila* (Figure 5.4) and the MICs were similar for all four methods: BYE-α and LASARUS (MIC_{50}=16µg/mL and MIC_{90}=32µg/mL); agar dilution on BCYE-α (MIC_{50}=32µg/mL and MIC_{90}=32µg/mL) and reduced MIC for gradient strip tests (MIC_{50}=2µg/mL and MIC_{90}=4µg/mL).

The MIC ranges for these three antimicrobials were very tight, generally entirely contained within a 3-4 serial dilution range for all isolates. However, a greater strain to strain MIC variation was observed for azithromycin Figure 5.5. Overall concordant values were again observed for LASARUS and BYE-α (MIC_{50}=0.032µg/mL and MIC_{90}=0.064µg/mL) relative to significantly increases BCYE-α MIC values (MIC_{50}=0.256µg/mL and MIC_{90}=0.256µg/mL) and consistently elevated values for gradient strip tests which gave 2 fold elevated results (MIC_{50}= 0.064µg/mL and MIC_{90}=0.128µg/mL).

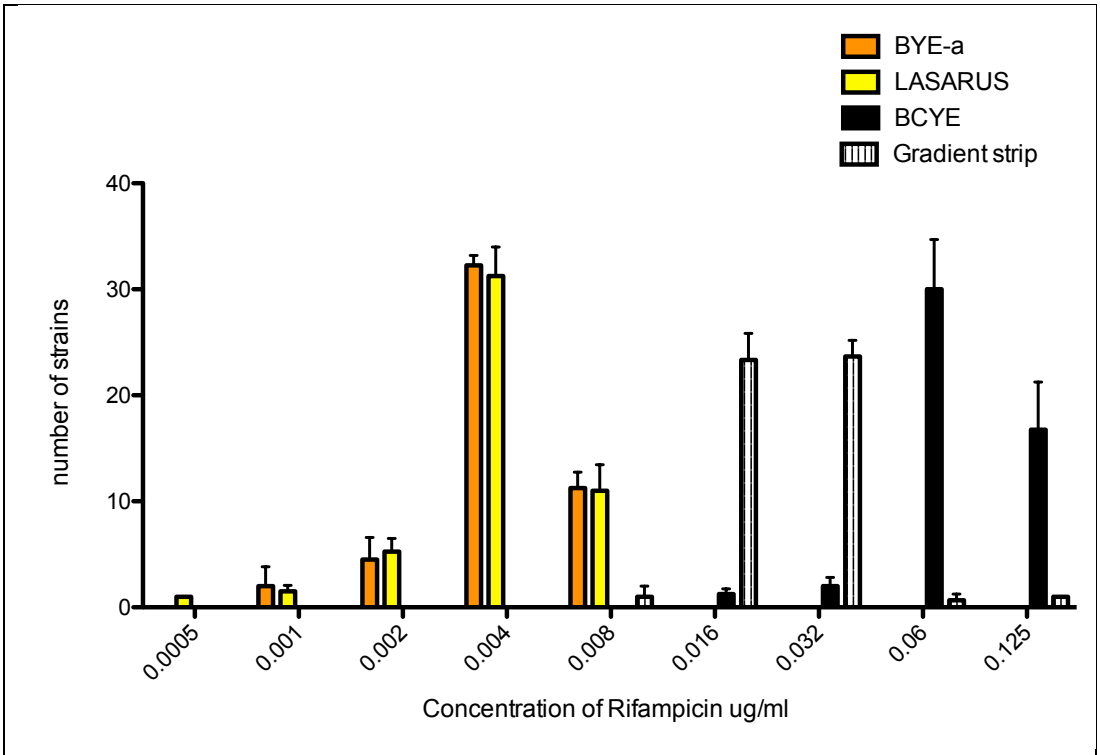


Figure 5.2 Rifampicin MIC determination of 50 *Legionella* pneumophila

Antimicrobials were diluted in liquid BCYα (Orange; reference BMD method) compared to solid media LASARUS (Yellow) and BCYE-α (agar dilution-Black, gradient strip-White). Data represents the mean ± standard deviation for four replicates. (Gradient strips were replicated in triplicate).

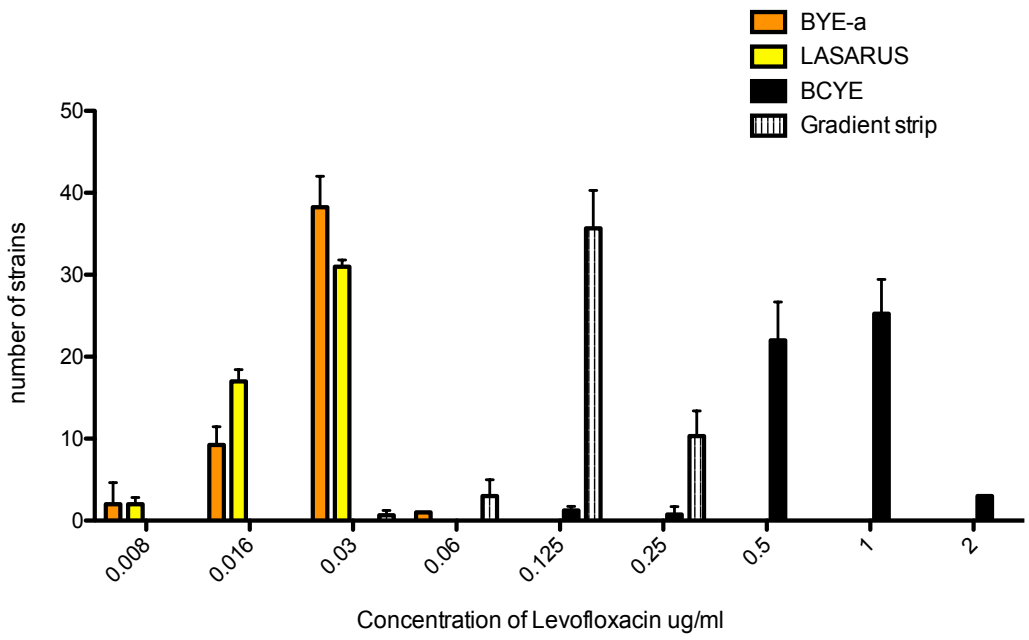


Figure 5.3 Levofloxacin MIC determination of 50 *Legionella* pneumophila

Antimicrobials were diluted in liquid BCYα (Orange; reference BMD method) compared to solid media LASARUS (Yellow) and BCYE-α (agar dilution-Black, gradient strip-White). Data represents the mean ± standard deviation forfourreplicates. (Gradient strips were replicated in triplicate).

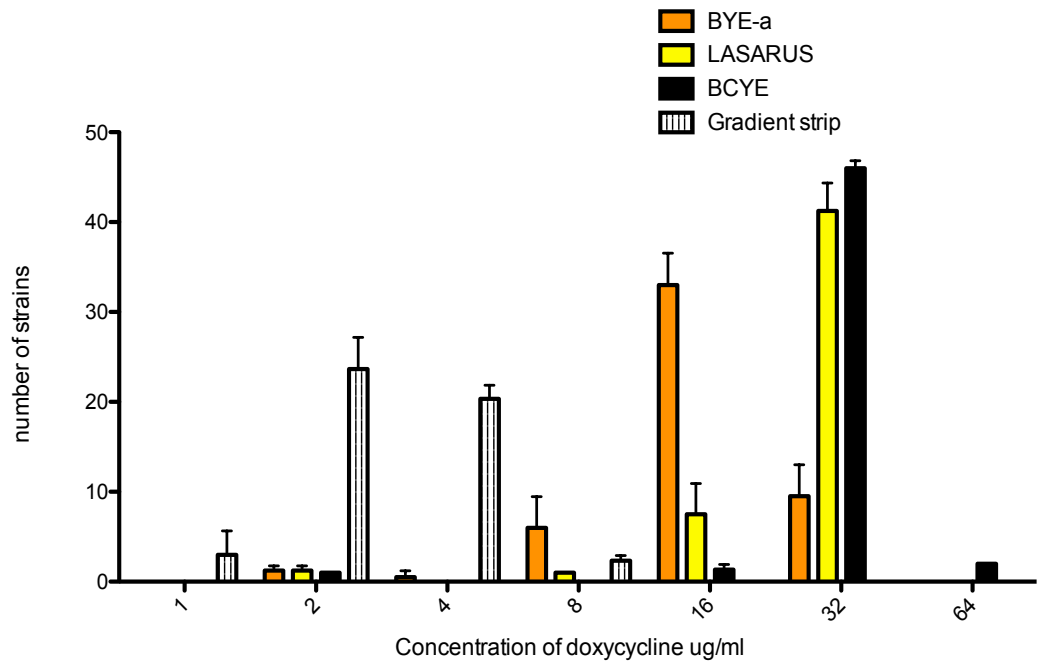


Figure 5.4 Doxycycline MIC determination of 50 *Legionella* pneumophila

Antimicrobials were diluted in liquid BCYα (Orange; reference BMD method) compared to solid media LASARUS (Yellow) and BCYE-α (agar dilution-Black, gradient strip-White). Data represents the mean ± standard deviation forfourreplicates. (Gradient strips were replicated in triplicate).

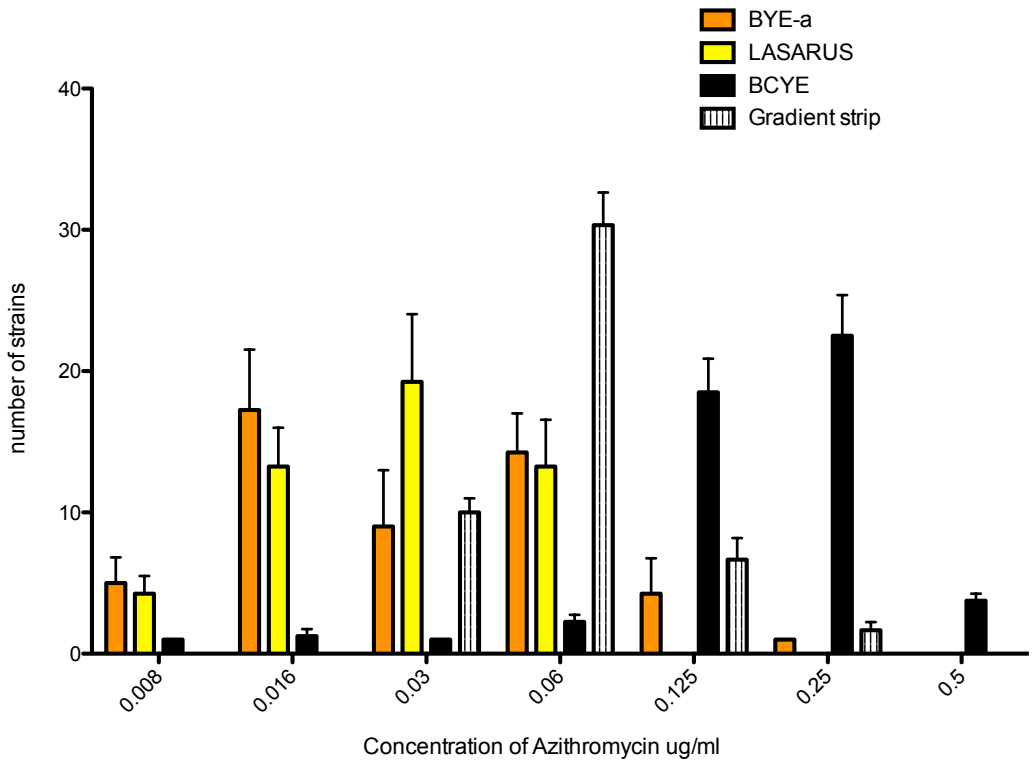


Figure 5.5 Azithromycin MIC determination of 50 *Legionella* pneumophila

Antimicrobials were diluted in liquid BCYα (Orange; reference BMD method) compared to solid media LASARUS (Yellow) and BCYE-α (agar dilution-Black, gradient strip-White). Data represents the mean ± standard deviation forfourreplicates. (Gradient strips were replicated in triplicate).

I noticed here that there was a wide range of MICs for azithromycin regardless of the method chosen to test. As such I was interested in determining the underlying mechanism of this range and whether the cause of the increased MICs was due to the known resistance gene (*lpeAB*). Closer analysis (limited to just one method of MIC determination= BYE-α), showed a range between <0.008 and 0.25µg/ml. I took 28 isolates spanning the ranges: 18 had MICs at or above 0.06µg/ml and 10, had MICs at or below 0.03µg/ml; these were then whole genome sequenced on an Illumina MiSeq to investigate the presence of any potential known resistance genes.

5.2.3 Whole genome sequence analysis of azithromycin susceptible and resistant strains.

Five mechanisms of macrolide antimicrobial resistance were explored by examining the WGS for the strains of *L. pneumophila*. Macrolide resistance-mediating mutations in the 23S rRNA gene are challenging to analyse as SPAdes often fails to correctly assemble the three separate operons of near identical sequence, however no mutations or sequence heterogeneity at position 2058 or 2059 (*Escherichia coli* numbering) of the *rrl* were identified. Furthermore, no mutations in the ribosomal accessory protein genes *rplD* (protein L4) and *rplV* (protein L22) were found. The 23S rRNA methylase genes *ermA*, *ermB*, *ermC*, and *ermF*, the macrolide phosphotransferase gene *mphA*, and the macrolide esterase genes *ereA* and *ereB* were not observed in any of the strains. Furthermore, no presence of the efflux pump genes *mefA/E* were observed in any of the strains. Analysis of isolates with MICs >0.06µg/mL using BYE-α media revealed that five of the highest MIC isolates tested contained the *lpeAB* efflux pump gene. None of the 18 isolates with MIC <0.003µg/mL (tested on BYE-α), which were sequenced contained the *lpeAB* efflux pump gene (Figure 5.6).

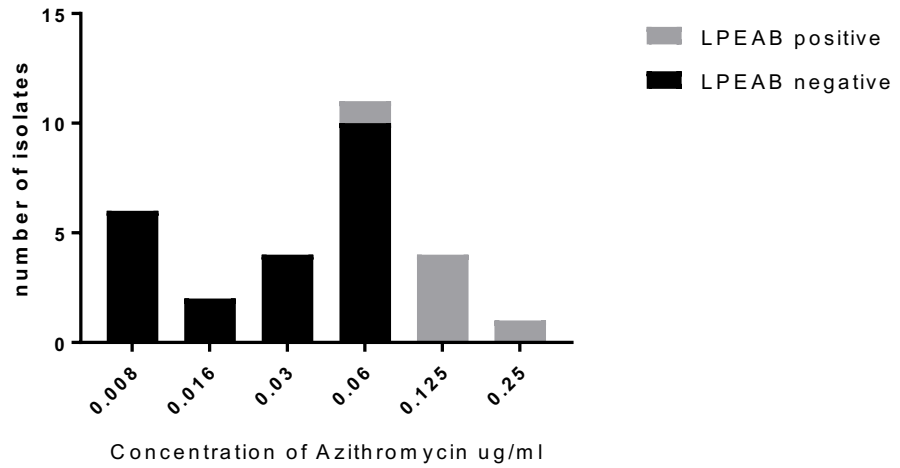


Figure 5.6 Investigation of *lpeAB* positive and negative isolates
A subset of 28 isolates spanning the range of azithromycin concentrations for BYE-α whole genome sequenced and analysed for *lpeAB*, five instances of the gene were found all in the higher MIC ranges.

5.2.4 Comparison to of MIC modal deviation from gold standard BMD to LASARUS, BCYE-α agar dilution and gradient strip test.

When considering BMD with BYE-α as the gold standard; the average modal deviation of other tested media against this highlights the overall similarity of BMD and LASARUS to within one serial dilution deviation, further emphasising the inaccuracy of BCYE-α with an MIC increase of up to five serial dilutions above gold standard. Gradient strip tests on BCYE-α were again elevated, showing 2-3 serial dilution deviations from BYE-α BMD.

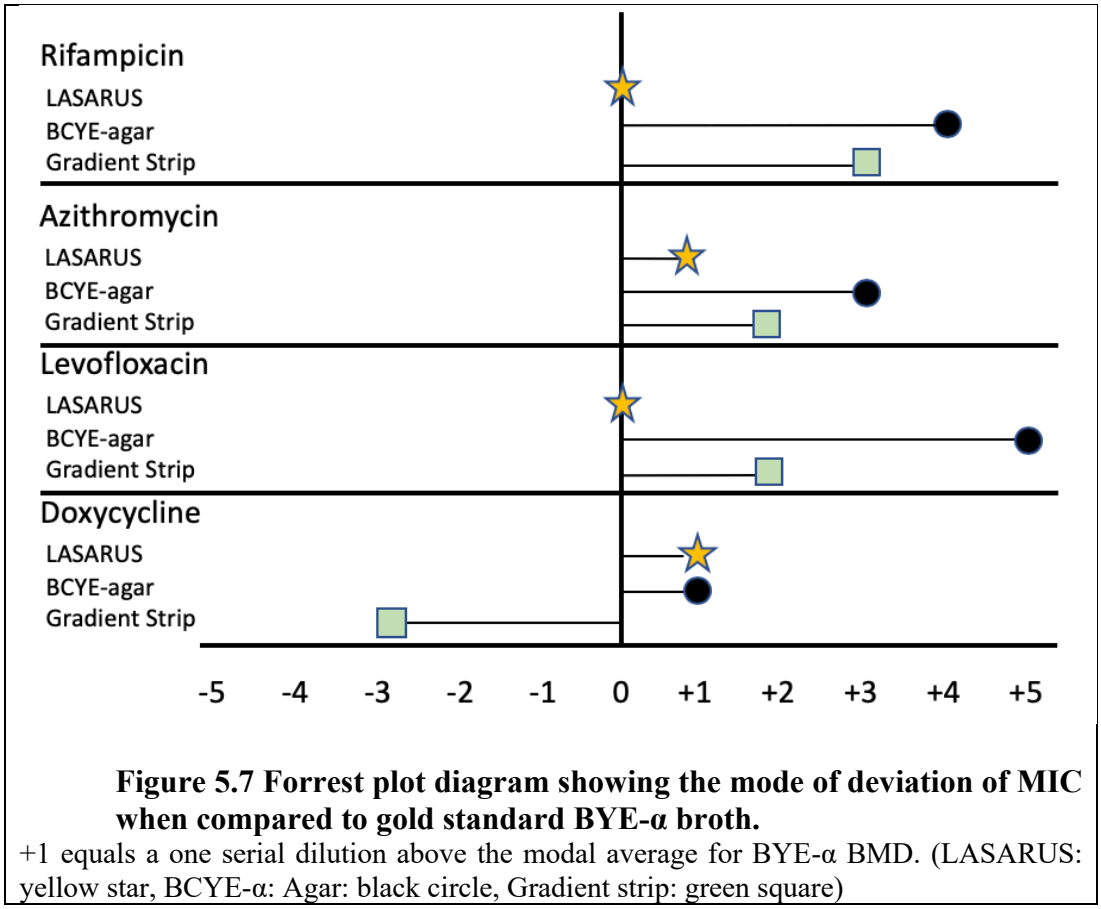


Figure 5.7 Forrest plot diagram showing the mode of deviation of MIC when compared to gold standard BYE-α broth.

+1 equals a one serial dilution above the modal average for BYE-α BMD. (LASARUS: yellow star, BCYE-α: Agar: black circle, Gradient strip: green square)

5.3 Discussion

Current guidance from EUCAST recommends users should determine MICs using antimicrobial gradient strips on BCYE-α for Legionellaceae. ECOFF values have not been assigned and there is an absence of agreed international guidelines to determine the resistance of any clinical or environmental *Legionella spp.* This has resulted in multiple methodologies providing a wide range of results in the literature as can be seen in the supplementary figure in (Portal et al. 2021b) or in the appendix, without standardisation, this continues to confound the definition between antimicrobial resistance and sensitivity for this species.

In line with other bacterial pathogens, I utilised BYE-α BMD as the gold-standard MIC methodology, which gave concordant MICs to serial agar dilution in LASARUS. Gradient Strips gave the next closest, but moderately elevated, MIC values. However serial antimicrobial dilution in BCYE-α agar, gave highly discordant and elevated MICs, with greater variability in the MIC range determined. Unfortunately, gradient strips are incompatible with the formulation of LASARUS plates as the zone of inhibition extended well beyond the boundaries of the strip. On LASARUS extensive antibiotic diffusion occurred irrespective of humidification of incubation. For each of these experiments' gradient strips from the same batch overlaid on *Legionella pneumophila*-inoculated BCYE-α plates run in parallel performed as expected. The reason why LASARUS has increased diffusion capacity is not clear and could be due to moisture content in the formulation promoting diffusion. A potential modification could be to increase the agar concentration from 1% to between 2-3% and see if this caused a reduction in moisture and therefore better tolerance of a gradient strip.

Interestingly, the chelating effect of activated charcoal, elevating MIC determination, has been reported previously (Ruckdeschel and Dalhoff 1999; Nielsen et al. 2000; Bruin et al. 2012) and while acknowledged it has generally been disregarded as a problem in the absence of solid culture media without charcoal, despite the impact on result accuracy. Garcia *et al* found equally discrepant MICs when comparing antimicrobial dilution series in liquid BCYα relative to incorporation into solid BCYE-α (García et al. 2000).

When evaluating an individual medium, all our isolates were inhibited within a distribution of 3-6-fold dilutions, similar to that found previously (Bruin et al. 2012), and showed relatively little overall isolate variation within this data set. However, when comparing combined data across all four methodologies in this study, the MIC distribution ranged between 6- to 9-serial dilutions, due to the disparity within the four investigated methods (Figure 6). This highlights the importance of a systematic comparison, with methodologies giving widely discordant results, especially those acquired by serial agar dilution in BCYE-α.

Beyond the advantages of being able to perform high-throughput testing on solid medium with multipoint inoculation, the translucent nature of LASARUS agar gave the additional advantage of safety and reduction in potential contamination when reading results, as opaque BCYE-α and 96-well plates for BMD require removal of lids due to condensation to determine culture growth. LASARUS agar would be amenable to measurement using automated optical systems, which was not attempted but could be implemented in a high throughput investigation. BMD based methodologies are uniformly defined as the gold standard reference methodology for the determination of MIC for most other bacterial species (e.g. CLSI guidelines M100, M43A, EUCAST.). However, in practice BMD is labour intensive and in assays

requiring incubation for ≥24 hours, imparting a much greater risk of interference by contamination relative to other bacteria that can be evaluated after 16-24 hours of growth. Furthermore, slower growth rate for Legionellaceae and manual determination of turbidity to confirm growth adds a greater degree of subjectivity for this bacterial species when performing AST.

Levofloxacin MICs investigated through BMD reported by Higa *et al.* showed a similar range of 0.004-0.125µg/mL compared to our findings of 0.008-0.125µg/mL for BMD and 0.008-0.06µg/mL for LASARUS (Higa et al. 2005). Our MICs determined by gradient strips were moderately elevated in comparison (0.03-0.25µg/mL). Furthermore, while our MICs were significantly elevated for BCYE-α agar dilution compared to all three presented methodologies with MIC_{50}=1µg/mL and MIC_{90}=2µg/mL; these results are still similar to the reported results by Martin *et al.* who showed MIC_{50}=1µg/mL to 41 *Legionella* spp. by BCYE-α serial dilution methods (Martin et al. 1996).

MIC determination for rifampicin by gradient strip tests carried out by Marques and Piedade showed an MIC_{50}=0.023µg/mL, concordant to the values obtained using gradient strips in this study (MIC_{50}=0.016µg/mL) (Marques and Piedade 1997). Agar dilution on BCYE-α of only 30 *Legionella* strains carried out by Edelstein showed MIC ranges between 0.03-0.06µg/mL, falling two-fold dilutions of those found in this study 0.016-0.125µg/mL (Edelstein 1991). Reported BMD ranges for rifampicin vary from 0.00012-0.03µg/mL across three different publications (Gómez-Lus et al. 2001; Vandewalle-Capo et al. 2017; Wilson et al. 2018), and Gomez-Lus *et al* (Gómez-Lus et al. 2001) showed an MIC_{50}=0.004µg/mL for 140 *Legionella pneumophila* identical to our findings.

MICs reported for doxycycline gradient strips against 32 isolates showed a similar MIC_{50} and MIC_{90} of 2µg/mL and 3µg/mL, respectively, compared to our MIC_{50}=2µg/mL and an MIC_{90}=4µg/mL (Marques and Piedade 1997). The authors noted an increase in MIC during incubation on BCYE-α from 8µg/mL at 48 hours to 32µg/mL at 72 hours. This highlights that there is a stability issue in antibiotics which are bacteriostatic rather than bactericidal which when reading results at different times may lead to variations in results, this further emphasises the importance of standardisations.

The unusual finding in this study that doxycycline MICs were lower for gradient strips than all other methodologies, in contrast to the findings for all other antimicrobials I tested, this could be could be due to reduced dispersion of doxycycline in the media, which was previously hypothesised by Isenman *et al.* for tetracycline as they are such large molecules 444.4g/mol−1 (Isenman et al. 2018).

Azithromycin MICs on BCYE-α by gradient strips in this study showed MIC_{50}=0.064µg/mL and MIC_{90}=0.125µg/mL, comparable to MIC_{50}=0.047µg/mL and MIC_{90}=0.25µg/mL for 100 isolates from Italy (De Giglio et al. 2015), and ranges reported by others including 0.016-4µg/mL and 0.06-4µg/mL (Vandewalle-Capo et al. 2017; Miyashita et al. 2018). Further, the BMD MIC range of 0.008-0.125µg/mL in this study was dissimilar to that reported by Sharaby *et al.* (Sharaby et al. 2019) who found an MIC range of 0.038-1µg/mL. The greater MIC range for azithromycin (relative to tight ranges for other antimicrobials) is reported and can be explained by presence of the gene *lpeAB*, (Vandewalle-Capo et al. 2017; Natås et al. 2019). Consistent with this finding, *lpeAB* was present in 5/10 *L. pneumophila* isolates with MICs above 0.06µg/mL in our study and completely absent from all sequenced strains (n=18) which had MICs below 0.016µg/mL.

This is the first identification of this gene conferring decreased susceptibility to azithromycin in *L. pneumophila* isolates from English and Welsh isolates.

5.4 Conclusion

BMD based AST methodology should be the internationally agreed gold standard for the determination of MIC for *Legionella* spp. This method is well-established for multiple bacterial pathogens conferring accurate inoculum concentration and resultant MIC (CLSI 2020; EUCAST 2021). Agreed medium formulation, incubation length and inclusion of ATCC/NCTC-deposited reference strains should be included in the international standardisation of methodology as per both EUCAST and CLSI guidelines.

The use of serial agar dilution using BCYE-α or gradient strip overlay on BCYE-α gave significantly elevated and more variable MIC results than gold standard BMD, and therefore should be discontinued in *Legionella* spp. MIC determination. However, for ease of use and adaptation to automation, the new commercially available LASARUS solid medium was the only method that gave results concordant to within one serial dilution to BMD for all antimicrobials tested. Therefore, AST determination by antimicrobial agar dilution in LASARUS should be considered to have equal validity to BMD methodologies. Future work may involve developing a LASARUS formulation which would work with gradient stripes.

This study is the first to identify the *lpeAB* gene in UK isolates and confirmed that presence of this gene consistently decreased susceptibility to azithromycin in *L. pneumophila* isolates. This supports susceptibility determination in cases with persistent infection and for continued surveillance to identify emerging resistance trends in *L. pneumophila*. However, the absence of international guidelines and breakpoints for Legionellaceae makes routine MIC determination in clinical cases

difficult to interpret and urgently requires international consensus. These issues have been raised in my international position paper (Portal et al. 2021b).

6 Results Chapter 3 The antimicrobial susceptibility profile

The antimicrobial susceptibility profile of the UKHSA clinical *Legionella* archive from 2003 to 2019.

6.1 Introduction

This chapter investigates the antimicrobial susceptibility profile of *Legionella*, using LASARUS agar containing serial dilutions of antibiotic concentrations in combination with a multipoint inoculator to high throughput screen the majority of the collection of *Legionella* spp. within the UKHSA archives from 2003 to 2019. This enabled MIC_{50}, MIC_{90} and ranges to be determined. This data, when analysed highlighted isolates, which had elevated susceptibility, consistent with suspected resistance profiles, and these were then further explored. WGS was then carried out using Illumina MiSeq and ONT MinION to create sealed genomes in an attempt to determine the underlying mechanisms of these putative resistant strains. Due to the significant differences in the 'mode of action' for different antibiotic classes investigated, as well as published methods of resistance for the antibiotics screened, this chapter is divided into subchapters. Each antibiotic class is examined individually: each presented with a brief introduction, results for MIC and ranges, with re-evaluation of those with higher MICs, details of potential mechanisms of resistance, followed by antibiotic specific discussion. Those isolates found to have elevated MICs for multiple antibiotics are re-visited in depth at the end of the chapter. This includes a phylogenetic analysis of all putative resistant isolates, both with single elevated and multiple elevated MICs. The antibiotics investigated were selected for their array of mechanisms of action and diverse classes to ensure a wide coverage, not just

investigations limited to common treatment options. The table below shows the antibiotics used for the screening in this chapter.

Table 30 Antibiotics tested and their method of cellular disruption.

Subsection	Antibiotic	Drug class	Drug target
6.2	Gentamicin	Aminoglycoside	Inhibition of protein synthesis 30S, A site
6.3	Ampicillin	Beta-lactam	Disruption of cell wall synthesis
6.4	Ciprofloxacin	Fluoroquinolone	Blocks gyrase/topoisomerase
6.4	Levofloxacin	Fluoroquinolone	Blocks gyrase/topoisomerase
6.5	Tetracycline	Tetracycline	Prevent binding of new incoming aminoacyl-tRNA, 30S
6.5	Doxycycline	Tetracycline	Prevent binding of new incoming aminoacyl-tRNA, 30S
6.6	Chloramphenicol	Phenicol	Blocks peptidyl transferase in ribosome, 50S
6.7	Azithromycin	Macrolide	Blocks ribosomal exit tunnel, 23S rRNA
6.8	Rifampicin	Ansamycin	Inhibition of RNA polymerase

6.2 Gentamicin

6.2.1 Results

Initial screening of 2,156 isolates against gentamicin examined a range between 0.03µg/mL and 1µg/mL.

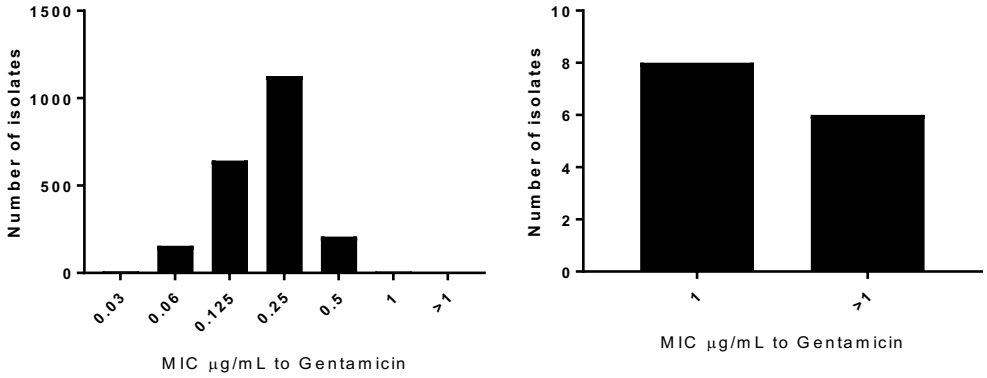


Figure 6.1 A gentamicin MIC determination for n=2156 *Legionella* spp. Methods as per 3.4. The graph on the right only shows the findings exclusively for MIC1µg/mL and >1µg/mL isolates as they are too few to be observed at the scale of the graph on the left.

The distribution of MICs above is consistent with a "normal" or Gaussian distribution. These results give an MIC_{50} and an MIC_{90} of 0.25µg/mL, this shows a very narrow distribution range of inhibitory concentrations for gentamicin. All isolates with an MIC that exceed 0.5µg/mL of gentamicin, which represented less than 0.3% of the tested isolates, were subjected to further investigation. These were re-assessed at a higher concentration range (between 1 and 16µg/mL) to determine a more accurate MIC against gentamicin.

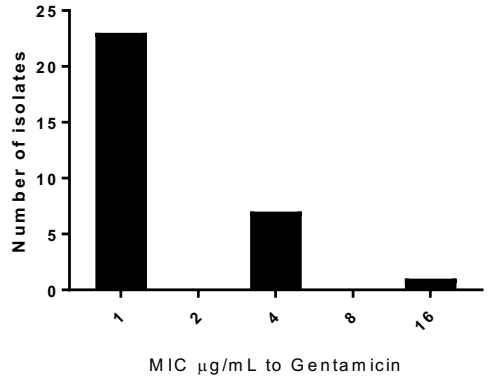


Figure 6.2 Gentamicin MIC determination for n=31 *Legionella spp*
Follow up experiment showing high MIC isolates as per methods 3.4.

Of the 31 isolates that were re-evaluated 23 had an MIC of 1µg/mL and these were not further investigated. Seven isolates had an MIC of 4µg/mL and one isolate had an MIC of 16µg/mL, which represents a 16-fold and a 128-fold increase compared to the MIC_{90} of the whole cohort respectively. These eight strains represent a clear break from the normal distribution seen in the initial analysis and as such were further investigated.

An individual colony was picked from the antibiotic negative control plate, (as they had the best growth), scaled up and extracted using the Qiagen QiaCube. These were then Illumina sequenced, MiSeq). Of the eight isolates to be sequenced, two were not *Legionella* species and excluded (both had MICs of 4µg/mL). This would have been identified using a MALDI-ToF on colonies but due to the COVID-19 pandemic, this equipment was inaccessible and therefore these were not further investigated. The remaining six isolates are shown in the table below.

Table 31 Sequenced isolates with reduced sensitivity to gentamicin

ID	ST	Clinical linkage	source	Location	isolation date	MIC to gentamicin
45-36	37	X	Clinical	England	21/10/2016	16

49-03	74	Y	unknown	Unknown	11/09/2017	4
49-08	1012	K	Clinical	Greece	11/09/2017	4
49-09	42	K	Clinical	Greece	11/09/2017	4
51-09	47	L	Clinical	England	16/11/2017	4
52-38	256	M	Clinical	Unknown	15/01/2018	4

As these six isolates were multidrug resistant (MDR) (resistant to at least three antibiotics) as detailed in the sections below, further in-depth investigation will be detailed in section 6.9.

6.2.2 Discussion

In creating an MIC database for over 2100 *Legionella* spp. isolates I have been able to create an MIC_{50+90} to gentamicin 0.25µg/mL and >1µg/mL, respectively. Whilst also determining the point at which an isolate becomes resistant to gentamicin at 4µg/mL. As of yet there are no papers published which thoroughly investigate gentamicin sensitivity on *Legionella spp.* A PubMed search using the terms '*Legionella*' and 'gentamicin' produces 39 papers ranging from 1978 to 2021 (date investigated: 11/2021). 18/39 of these were papers using gentamicin to kill extracellular *Legionella* in cell cultures and 16 of these were irrelevant to the topic. The remaining five represent the only published investigations into gentamicin's MICs on *Legionella*.

Gibson and Fitzgeorge in 1983 showed an MIC of 0.5µg/mL to gentamicin in testing a single *Legionella* isolate but noted its lack of cellular penetration in *in vivo* models (Gibson and Fitzgeorge 1983). An investigation into the causative agent of the 1982 Pittsburgh pneumonia agent, which turned out to be the *Legionella micdadei* (SIC), determined that the MIC to gentamicin was 0.25µg/mL using a broth dilution method (Dowling et al. 1982). This is in concord with my data. A follow up *in vivo* experiment accessing a guinea pig model found that; while high levels of gentamicin was not curative it did reduce infection and the sample group had a higher survival rate, which suggests that killing extracellular *Legionella* could act as an effective combination therapy (Pasculle et al. 1985). A 1987 paper on *Legionella* showed a single isolate with an MIC of 0.39µg/mL this falls well within my tested MIC range for gentamicin(Havlichek et al. 1987). A 1978 experiment, which investigated MIC of *Legionella* in egg yolks (the initial culture method) found a gentamicin MIC of

0.25µg/mL (Lewis et al. 1978). These papers all evaluate *Legionella* against gentamicin, and all found results which fit within the investigated MIC range.

The only known aminoglycoside resistant gene identified to date is consistently found in *Legionella* species is the aph(9)-Ia gene, first described by Suter *et al* (Suter et al. 1997). This gene confers resistance only to spectinomycin (not technically an aminoglycoside, but an aminocyclitol). This gene appears to have originated in *Legionella* or a recent common ancestor based on its genomic context (ie. no nearby insertion sequences and a matching GC content). Intrinsic resistance conferred by this gene is possibly explained as a by-product of the aquatic living environment of natural *Legionella* in constant contact with antibiotic producing bacteria such as Streptomyces (Suter et al. 1997; Thompson et al. 1998).

My work has evaluated the MIC_{50+90} of gentamicin providing the first large scale analysis of resistance to gentamicin within *Legionella*. A literature review suggested that few others have carried out thorough investigation into gentamicin and those that have were very small in scale and only between 1978 and 1987. The results all agreed with the ranges we found (0.06-0.5µg/mL).

This work has allowed a determination of the threshold of resistance to gentamicin being 4µg/mL, which represents 4-fold higher than 1µg/mL, where 99% of tested isolates tested were inhibited. As gentamicin is not clinically used in the role of *Legionella*, this work has not been taken further but its use in setting aminoglycoside thresholds for resistance is clear.

6.3 Ampicillin

6.3.1 Results.

2,175 isolates were screened against ampicillin testing a range between 1µg/mL and 32µg/mL.

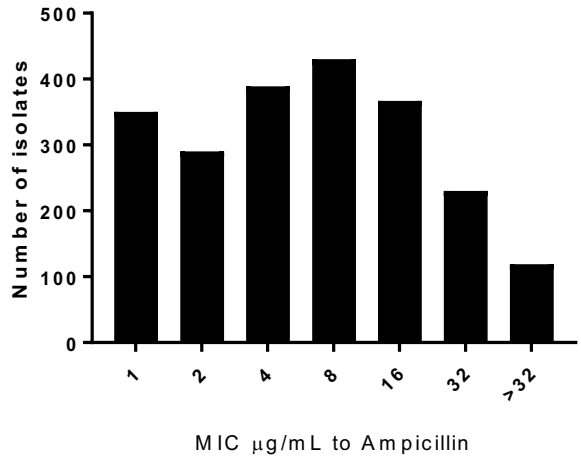


Figure 6.3 Ampicillin MIC determination for n=2175 *Legionella spp.*
Showing MIC against ampicillin as per methods 3.4

Unlike the results detailed in section 6.2 for gentamicin, a very broad range of MICs to ampicillin was observed, but still maintain a "normal" or Gaussian distribution appearance. The increased MIC range is suggestive of the potential presence of resistance genes. The MIC_{50} for ampicillin was 8µg/mL and the MIC_{90} was 32µg/mL. The 119 isolates that had an MIC above 32µg/mL were re-investigated and assessed against a higher concentration range of ampicillin: 16-128µg/mL.

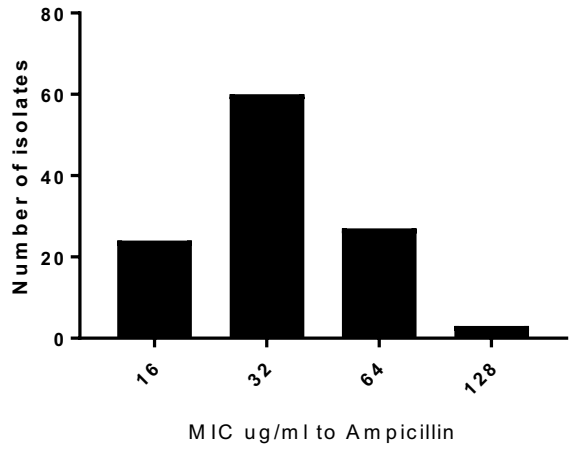


Figure 6.4 Ampicillin MIC determination for n=114 *Legionella spp.*
Follow up experiment showing high MIC isolates as per methods 3.4

Of the n=114 isolates that were rescreened against a higher range of ampicillin (five isolates were repeated and not included as they were found to be duplicates within the strain collection). Twenty-four isolates were found to have an MIC=16µg/mL and 60 were found to have an MIC=32µg/mL, this represented a minor 2-fold variation that is common to biological replicates of dilution-based MIC experiments so not significant. More interestingly 27 isolates had an MIC=64µg/mL and three isolates had an MIC of 128µg/mL. It appears that the three isolates with a 128µg/mL MIC could be considered in the 2.5% outermost part of a Gaussian distribution, rather than a pronounced shift imparted by the presence of a resistance gene, as they only have a 4-fold increase as compared to the MIC_{90}. Regardless, the isolates with the highest MIC to ampicillin were further investigated for resistance mechanisms. An individual colony was picked from the antibiotic negative control plate, as they had the best growth, scaled up and extracted, sequenced and shown in Table 32.

Table 32 Sequenced isolates with high resistance to ampicillin

ID	ST	Clinical linkage	Source	location	date isolates	Ampicillin MIC
45-24	1	U	Clinical	Greece	21/10/2016	128
49-13	84	W	Clinical	Greece	04/09/2017	128
45-61	23	V	Clinical	Unknown	08/09/2017	128

Isolate 45-24 was an ST1 clinical isolate, isolated in October 2016 with an MIC to ampicillin of 128µg/mL. This isolate was not resistant to any other screened antibiotics. Whilst attempting to determine the cause of the increased MIC, several beta lactamases were discovered. Firstly, these isolates contained the *loxA* gene, as reported by Avison and Simm in 2002 (Avison and Simm 2002). This matched with 100% identity to the *loxA* gene found in the Paris strain of *Legionella pneumophila* Sg 1. Isolate 45-24 also contained a plasmid, which was 149,372bp long, and contained two additional beta-lactamase genes. The first was a beta-lactamase serine hydrolase, which has a 100% identity to a serine hydrolase annotated in prototype Philadelphia monoclonal subtype of *L. pneumophila* Sg 1. Additionally, and more interestingly, was the presence of the $bla_{\text{oxa-18}}$ gene, which is a class D beta-lactamase, first reported in *Pseudomonas aeruginosa* in 1997, which has been shown to be effective against a broad range of penicillins (Philippon et al. 1997; Kalai Blagui et al. 2007). As such, this is the most likely cause of the increased MIC seen in this strain. This gene is significantly different from the *loxA* gene, as can be seen in the alignment below Figure 6.9, and equally dissimilar to *bla-oxa29* both of which are commonly found in *Legionella* species.

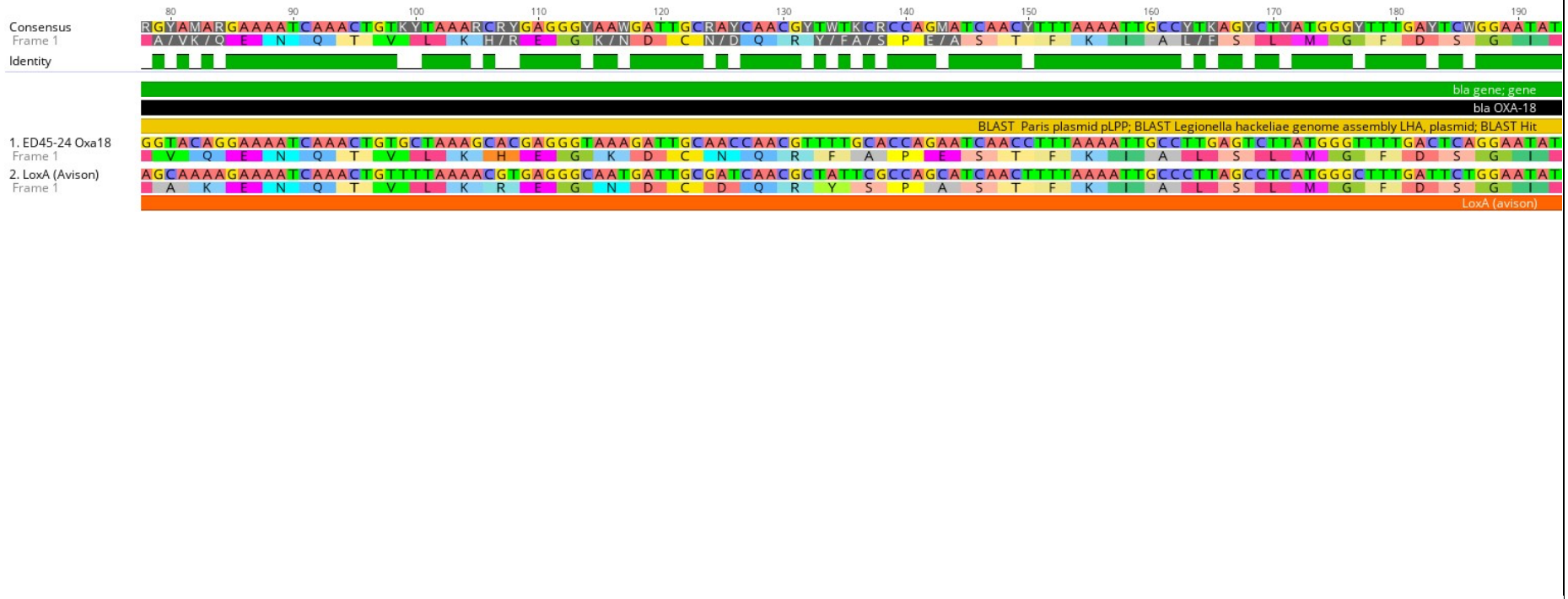


Figure 6.9 Alignment of *loxA* and OXA-18

Genious prime comparison of the first *loxA* gene from Avison *et al* and the Oxa-18 gene from 45-24 showing the significant discord between the two genes which share a Pairwise Identity of 77.8%.

Of interest 45-24 did not contain the plasmid-based *bla-$_{oxa29}$* gene, which is commonly associated with *Legionella* species (Franceschini et al. 2001). However, in the course of the investigation into *bla$_{oxa}$-$_{18}$* isolate 53-25, which was sequenced for its raised MIC to azithromycin, the *bla$_{oxa}$-$_{18}$* gene was discovered, while only having an MIC to ampicillin of 16µg/mL. Isolates 45-24 and 53-25 carried identical plasmids, with only three SNP differences. However, one of these variations mediated the mutation H292N in the second serine hydrolase beta-lactamase adjacent to Oxa-18. This may mediate a difference in MIC, but does not explain the elevated MICs for isolates 49-13 or 45-61, which lacked bla$_{oxa-18}$.

Isolate 49-13 is an ST84 clinical isolate, which is travel associated with Greece. This bacterium was isolated in September 2017 and has an MIC of 128µg/mL. Sequencing revealed the presence of LoxA, as well as an absence of *bla-$_{oxa29}$*, and no other beta-lactam resistance genes. Submission through ResFinder revealed only the presence of an aminoglycoside resistance determinant APH(9)-Ia at 100% identity and *loxA* (which is not present in ResFinder, but immediately recognisable as a gene with 78.4% identity to *bla-$_{oxa29}$*). The aminoglycoside O-phosphotransferase APH(9)-Ia, is an aminoglycoside resistance gene but has a very narrow range of efficacy, as it only mediates resistance to spectinomycin (an aminocyclitol rather than true aminoglycoside) and has no activity on other antibiotics.

Isolate 45-61 is an ST23 clinical isolate, which is of unknown origin. This sample was isolated in September 2017 and has an MIC of 128µg/mL. ResFinder analysis revealed only the presence of the spectinomycin resistance determinant APH(9)-Ia at 100%

identity and *bla-*$_{oxa29}$ at 78.4% identity (i.e. *loxA*). Phylogenetic analysis of the identified *bla* genes for all of these sequenced isolates is shown in Figure 6.5.

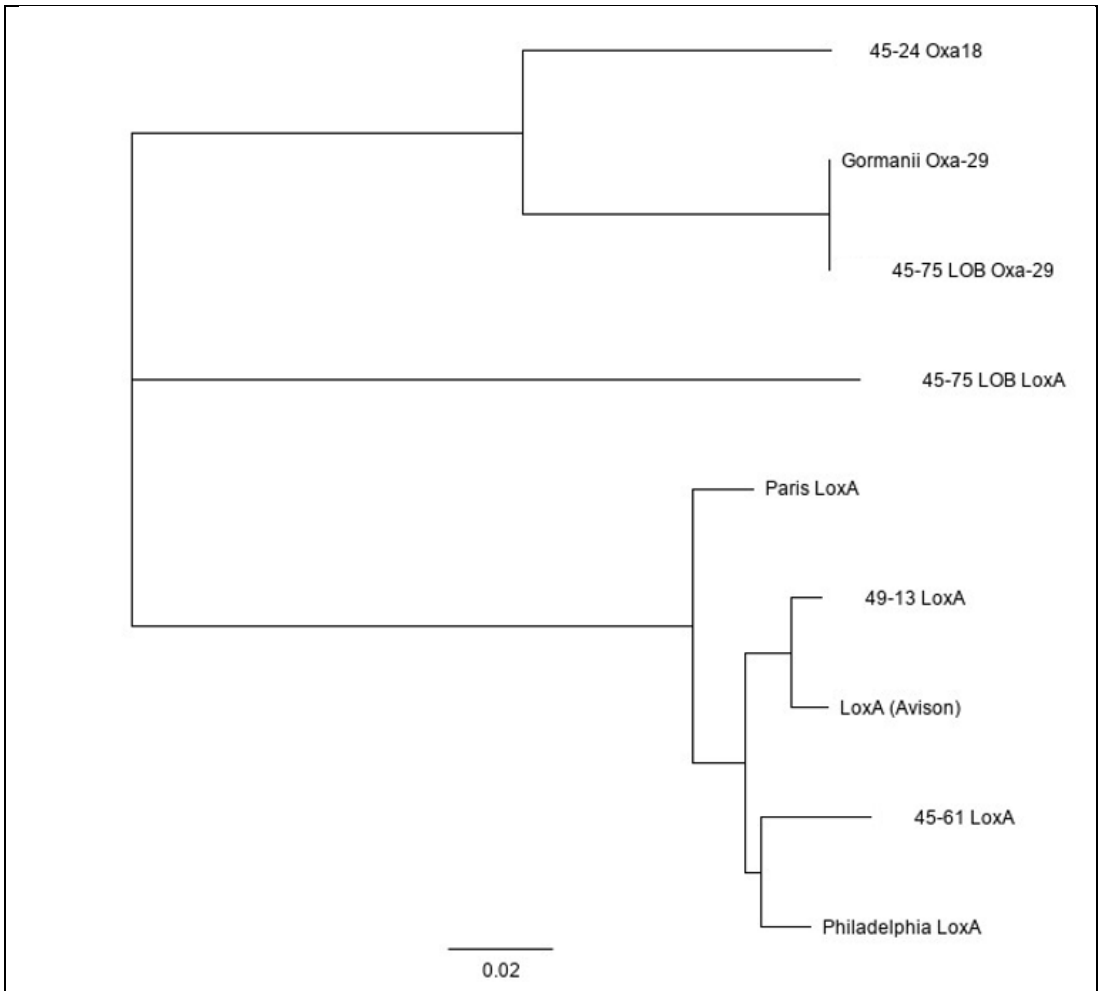


Figure 6.5 A gene phylogeny of the loxA and bla-oxa29

Figure 6.5 shows a gene phylogeny of the *loxA* and *bla-*$_{oxa29}$ using made usingGeneious Prime, a comparison of the various *Legionella* beta lactamases. This includes *Legionella gormanii bla*$_{oxa-29}$ and Avisons *loxA* the first publication of these resistance genes. This highlights the dissimilarity of *bla*$_{oxa-29}$ from *loxA* whilst highlighting the additional separation in 45-75, which is a *Legionella longbeachae* containing a different *loxA* gene.

This tree shows the 45-24 *bla*$_{oxa18}$ gene is very distinct in sequence from the other genes (except for identical sequence to *bla*$_{oxa18}$ gene from 53-25, which was not included) but is closer related to the original *L. gormanii bla-*$_{oxa29}$ gene than the *loxA* gene. The *loxA* gene from 49-13 maps closely to, but not identically to the *loxA* gene initially reported by Avison with only 4aa relative polymorphisms. While the *loxA* gene from isolate 45-61 most closely maps to the *loxA* gene found in *Legionella pneumophila* strain Philadelphia. Included on this tree are two separate *bla* genes from *L. longbeachae* isolate 45-75; one of which is identical to *bla-*$_{oxa29}$ from *L. gormanii* that was located on a plasmid as well as *bla*$_{OXA}$ gene that was located on the genome (referred to as LOB *loxA* on the tree) that is quite distinct from all other sequences examined. The *loxA* gene from the Paris strain is also included for reference.

While screening 31 isolates for inclusion in a manuscript looking at the presence and or absence of *lpeAB* for (Portal et al. 2021a). A distinct separation of ampicillin MIC between two variant groups was apparent. Analysis of the genomes showed the two resistance genes of interest, *bla-*$_{oxa29}$ at 100% homology as well as *bla-*$_{oxa29}$ homolog at ~77% homology (i.e. Avison's LoxA). Of the 31 isolates investigated n=14 (45%) possessed both *bla-*$_{oxa29}$ and *loxA* while the other n=17 (65%) only had the *loxA* gene.

This cohort of 24 isolates containing either both the *loxA* and the *bla-*$_{oxa29}$ genes (n=9) or only the *loxA* gene (n=15), were then further investigated to see if the presence of multiple beta lactamases would alter their resistance.

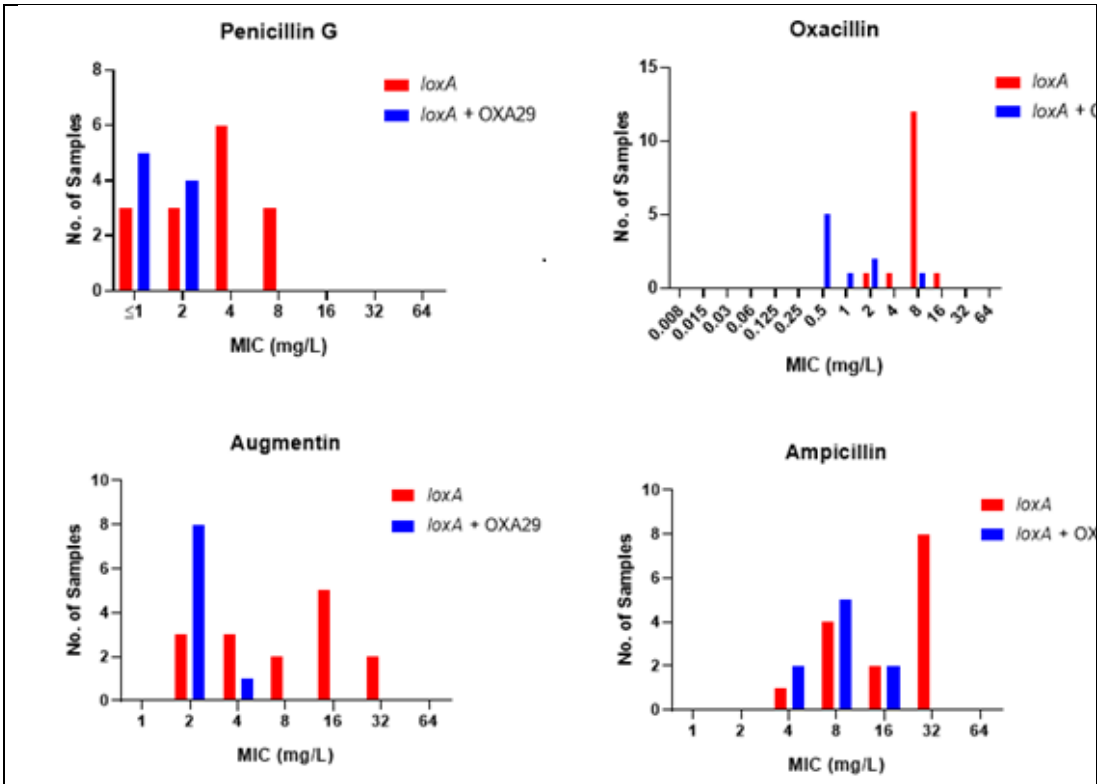


Figure 6.6 Graphs showing the separation of isolates by the presence of the *bla-$_{oxa29}$* gene.
(This work was done with George Steaman-Gay under my supervision).

Unpaired t-test between *loxA* and *loxA*+ *bla-$_{oxa29}$* groups showed that all antibiotics yielded significantly different results (p=<0.05) (GraphPad Prism V.7.04); unexpectedly showing the presence of both beta-lactamases resulting in a significantly lower MIC. This was most apparent in the oxacillin antibiotic test where the MIC difference was up to 32-fold lower for isolates containing *loxA* alone compared to isolates carrying both *loxA*+ *bla-$_{oxa29}$*.

6.3.2 Discussion:

This screening work showed both the prevalent level of intrinsic ampicillin resistance (as high as 128µg/mL) as well as the breadth of the range of ampicillin MIC, extending to susceptibility to ampicillin concentrations as low as 1µg/mL. The underlying cause of the high-level resistance is most likely due to the presence of genes of the *loxA* family (Avison and Simm 2002). This work has shown the MIC_{50} and the MIC_{90} of 8µg/mL and 32µg/mL respectively. Chasing down the highest MIC isolates with an MIC of 128µg/mL showed the presence of $bla_{oxa\text{-}18}$ conferring higher levels of resistance, but only in one of the three high MIC isolates, and the presence of $bla_{oxa\text{-}18}$ in an additional isolate with an MIC of only 16µg/mL. There was a mutation in the additional serine hydrolase adjacent to Oxa-18 for the isolate with the lower MIC. However, the underlying mechanism for those other two isolates with the highest MIC could not be further elucidated and given the clinical irrelevance of ampicillin to treatment, these isolates were not further investigated.

Edelstein and Meyer in 1980 showed an MIC_{50} of 2µg/mL and an MIC_{90} of 4µg/mL to 33 strains of *Legionella pneumophila* (Edelstein and Meyer 1980). These fell within my testing ranges observed in this study, but are 4-fold lower as carried out on BCYE-α. Ruckdeschel *et al,* which also investigated ampicillin MIC on BCYE-α showed a range of 0.06-8µg/mL with an MIC_{50} of 1µg/mL and an MIC_{90} of 2µg/mL on 60 isolates tested in 1984 (Ruckdeschel et al. 1984). The only report investigating ampicillin sensitivity on the current gold standard (BYE-α broth microdilution) was by Wilson *et al*, who investigated 92 isolates and determined the range to be 0.125-1µg/mL with an MIC_{50} of 0.5µg/mL and an MIC_{90} of 1µg/mL (Wilson et al. 2018). It is interesting that all above do not correlate well with our investigation, and all are between 4-8-fold lower than the LASARUS agar dilution has shown. This could be

down to the breadth of the isolates screened (n=2,175), relative to the low numbers (N=33-92) investigated by the other reports, or the difference in the methodologies used to determine MIC. As comparing ampicillin MIC between multiple methods was not examined for concordance in chapter 4, it is not possible to confirm the latter as the reason.

More interesting was previously unidentified apparent antagonism for beta lactam MICs when two separate beta-lactamase genes, *loxA* and *bla-*$_{oxa29}$, were present simultaneously. The expectation would that the presence of two β-lactamases to be better than one, especially as the second gene was previously shown to mediate resistance in *L. gormanii* (Franceschini et al. 2001). There may be an undiscovered mechanism of resistance to β-lactam antibiotics within the *loxA* only group, which is missing from the *bla-*$_{oxa29}$ plasmid carrying strains. However, it is more likely that genes responsible for controlling expression of these genes interfere with each other and this should be the focus of future investigations. It is of particular interest that simultaneous presence of *loxA* and Oxa-18 were not antagonistic, suggesting regulatory mechanisms found on the plasmid carrying *bla-*$_{oxa29}$ as the likely candidate. Follow up work to elucidate this should include a conjugation of the plasmid containing *bla-*$_{oxa29}$ into one of the *loxA* only strains to assess the change in MICs or plasmid curing of the *bla-*$_{oxa29}$ from a *loxA*+*bla-*$_{oxa29}$ strain for MIC comparison investigations. As this has no bearing on clinical treatment it was not further investigated, however an interesting concept and there are no other papers that have shown reduced antimicrobial capability with the addition of more resistance genes.

This work showed both the high level of intrinsic ampicillin resistance as well as the breath of the range of ampicillin resistance with isolates spanning 1-128µg/mL,

showing an MIC_{50} of 8µg/mL and MIC_{90} of 32µg/mL. Ampicillin is not clinically used in the treatment of *Legionella*, but it does set penicillin resistance thresholds.

6.4 Ciprofloxacin and levofloxacin

6.4.1 Results

2,028 and 2,208 isolates were screened against levofloxacin and ciprofloxacin, respectively, testing a range between 0.008µg/mL and 0.06µg/mL for both antibiotics.

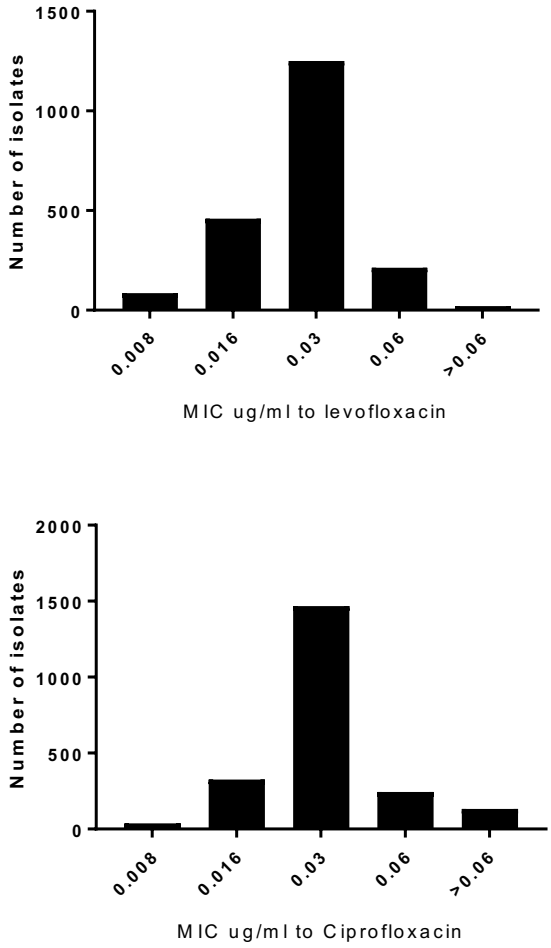


Figure 6.7 Levofloxacin and ciprofloxacin MIC determination for *Legionella spp. (*n=2028 and n=2208 respectively)
Showing MIC isolates as per methods 3.4 against both levofloxacin and ciprofloxacin

It is of interest to note that both these antibiotics overall appear to be very effective. Levofloxacin and ciprofloxacin both have an MIC_{50} of 0.03µg/mL and an MIC_{90} of 0.06µg/mL. However, when comparing the isolates that grew above that range, it shows that many more isolates achieved a higher MIC to ciprofloxacin than levofloxacin. Twenty isolates had an MIC above 0.06µg/mL for levofloxacin and 133

isolates for ciprofloxacin; representing less than 1% of the tested isolates for ciprofloxacin and 6% for levofloxacin. Repeating MIC determination for all isolates that >0.06µg/mL with a range between 0.125 and 2µg/mL for levofloxacin and between 0.03-2µg/mL for ciprofloxacin respectively are shown in Figure 6.8.

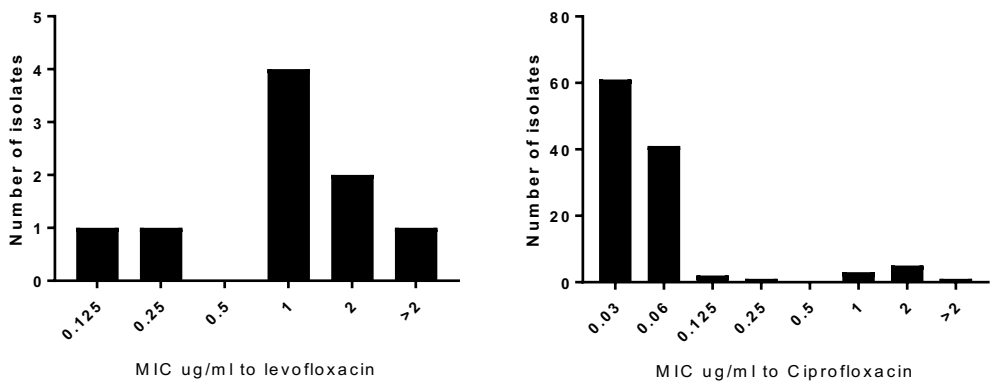


Figure 6.8 MIC of isolates with reduced susceptibility further determination for levofloxacin (n=9) and ciprofloxacin (n=114) *Legionella* spp.

Follow up experiment showing high MIC isolates for levofloxacin and ciprofloxacin isolates as per methods 3.4

Of the isolates investigated, there was a clear bimodal distribution of MICs separating susceptible isolates that were ±2 dilutions of the MIC_{90} of 0.06µg/mL (the tail end of the Gaussian distribution) and those that were above this range (≥1µg/mL). This essentially ensured a breakdown of isolates up to the 0.25µg/mL range for ciprofloxacin and levofloxacin, n=105 and n=20 respectively. There was still a single isolate for both levofloxacin and ciprofloxacin for which the MIC exceeding the new maximum MIC range of 2µg/mL. For ciprofloxacin there were three isolates with an MIC of 1µg/mL and five with an MIC of 2µg/mL. For levofloxacin there were four with MICs of 1µg/mL and one isolate with an MIC of 2µg/mL, respectively. All the same isolates were found to have elevated MIC for both fluoroquinolones and MIC

values were almost always identical for both antibiotics for each strain Table 33. Isolate 49-13 was also included as it had been sequenced for ampicillin resistance and also had a slightly increased MIC (0.125µg/mL) compared to the MIC_{90} for ciprofloxacin of 0.06µg/mL. These isolates were sequenced from the antibiotic free-plate – but all except 49-13 were multiple resistant strains, which will be discussed fully in section 6.9. Two isolates (52-38 and 51-09) were excluded at this stage as upon sequencing it was discovered they were contaminants (*Pediococcus* spp. and *Lactobacillus* spp., respectively).

Table 33 Information on fluoroquinolone resistant strains.

ID	ST	Clinical linkage	Source	Location	Date of isolation	Ciprofloxacin	Levofloxacin
52-38	256	m	Clinical	Unknown	15/01/2018	>2	>2
49-03	74	Y	Unknown	Unknown	11/09/2017	2	2
49-09	42	k	Clinical	Greece	11/09/2017	2	1
51-09	47	L	Clinical	England	16/11/2017	2	2
49-07	23	k	Clinical	Greece	11/09/2017	1	1
45-36	37	X	Clinical	England	21/10/2016	1	1
49-08	1012	k	Clinical	Greece	11/09/2017	1	1
49-13	84	W	Clinical	Greece	04/09/2017	0.125	0.06

An alignment of the few isolates that had reduced susceptibility to fluoroquinolones showed no variation in the respective QRDR that are expected for resistant isolates in GyrA, GyrB, ParC or ParE. Polymorphisms identified outside these regions, unique to 49-13 include GyrA P409S, ParC L133H and ParC M756I, but these are distant from the QRDR and unlikely to be important. Furthermore, no known fluoroquinolone resistance genes were identified by ResFinder. This is not wholly unexpected as QRDR mutations tend to confer resistance levels ≥8µg/mL. The observed reduced susceptibility of 2-4 fold increase in MIC is more in line with the presence of an efflux pump; however, no efflux pumps, which have been linked to fluoroquinolones resistance, were detected.

With the exception of isolate 49-13, which had a 2-fold increase in MIC (too low to be attributed to a QRDR mutation), the remaining isolates had elevated MIC for multiple antibiotics and are addressed further in section ‘Results of re-evaluation of strains with multi-drug resistance 6.9. It is possible that isolate 49-13 contained an as yet undiscovered efflux pump with limited homology to genes existing within the current ResFinder database, but further investigation was beyond the scope of time for this thesis.

6.4.2 Discussion

Comparing findings present in the literature of fluoroquinolone resistance *Legionella* spp., as previously shown, has the same limitations of differing methodologies that obfuscate direct comparison. However, fluoroquinolones are second only to azithromycin, as the focus of antibiotic susceptibility testing for *Legionella* spp., likely due to their clinical relevance.

There have been 27 studies investigating ciprofloxacin that passed the threshold for inclusion in the Portal *et al* comparison of concordance using the three methods of BCYE-α (agar dilution) (n=9), BCYE-α gradient strip (n=12) and BMD (n=6) (Portal et al. 2021b). As highlighted earlier, the inclusion of BCYE-α elevates the MICs compared to BMD. Average (mean rounded to nearest log) MIC_{90} for BCYE-α agar dilution from combined literature reports for gradient strip was 1µg/mL, whereas the six reports using BMD showed an MIC_{90} of 0.03µg/mL (n=5) and one showed an MIC_{90} of 0.06µg/mL. The BMD findings match (within 2-fold dilutions) with the results presented above.

With regards to levofloxacin, there are reports for the same three methods: BCYE-α (agar dilution) (n=3), BCYE-α gradient strip (n=10) and BMD (n=6). In general, the MICs are lower than for ciprofloxacin, perhaps as it is a later generation of fluoroquinolone. The results from the BCYE-α and gradient strip showed a mean MIC_{50} of 0.5µg/mL and MIC_{90} of 0.5µg/mL, compared to BCYE-α agar dilution with a mean MIC_{50} of 0.25µg/mL an MIC_{90} of 0.5µg/mL, respectively. These represent significant elevations compared to the results presented above.

The results from reports using BMD are more concordant, showing a mean MIC_{50} of 0.03µg/mL and a mean MIC_{90} of 0.06µg/mL: with a range of 0.004µg/mL to 0.25µg/mL. These results overlap the findings of the experiments displayed above

with MIC_{50} of 0.03µg/mL and MIC_{90} of 0.06µg/mL. The published comparison can be found at (Portal et al, 2021).

All previous studies were of fairly small scale, with the largest containing 271 strains and combined these total just over 2600 isolates. The addition of data for 2208 & 2028 isolates above means the current MIC knowledge for ciprofloxacin and levofloxacin against *Legionella* spp., using a method, which correlates well to that of the approved gold standard of BMD (Portal et al, 2021), has now almost doubled.

One article has presented a single case of ciprofloxacin resistance in a patient with significant ciprofloxacin exposure. The resistance was reported as 2µg/mL, representing only a 2-fold increase over the reported ECOFF value of 1µg/mL, determined by using E test gradient strips (Bruin et al. 2014). They attributed the resistance to a T83A GyrA mutation in the QRDR region. Idid not find any QRDR mutations in screening our resistant isolate 49-13. The only polymorphisms that were found (that were not additionally found in a small cohort of sequenced susceptible strains) were GyrA P409S, ParC L133H and ParC M756I. However, these positions have not been implicated in fluoroquinolone resistance in any other bacteria. Overall, the work carried out above has shown a lack of fluoroquinolone resistance and strongly implies that fluoroquinolones remain effective in the treatment of disease caused by *Legionella*.

6.5 Tetracycline and doxycycline

6.5.1 Results

Screening was initially carried out against tetracycline, investigating susceptibility for 2,188 isolates. A further review of therapeutic guidelines during these experiments highlighted differences in activity between tetracycline (never prescribed) and doxycycline (commonly prescribed). Therefore, analysis of the *Legionella* MICs was extended to include doxycycline for the final 536 isolates investigated, to give a more clinically relevant viewpoint and compare tetracycline and doxycycline susceptibilities.

The antibiotic concentration range tested was 8-128µg/mL for doxycycline and 32-128µg/mL for tetracycline, respectively.

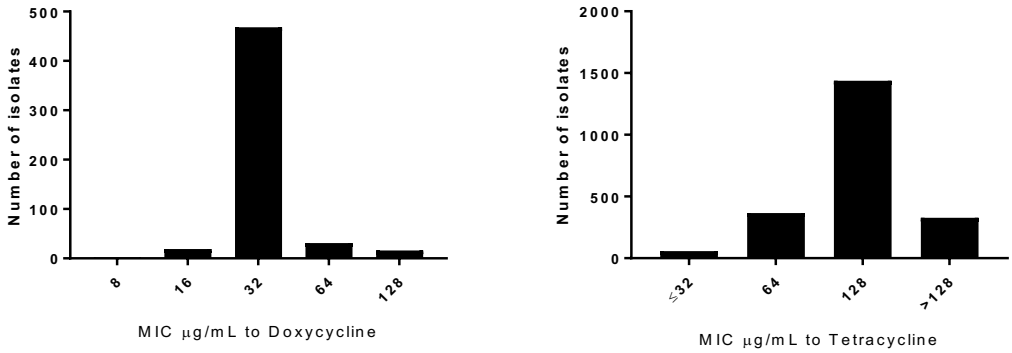


Figure 6.9 MIC determination for doxycycline (n=536) and tetracycline (n=2188) *Legionella spp.*

Initial experiments showing MIC isolates to both doxycycline and tetracycline as per methods 3.4

The MIC_{50} and the MIC_{90} for tetracycline was 128µg/mL. Of interest, doxycycline was around 4-fold more potent with an MIC_{50} and an MIC_{90} of 32µg/mL and a range of 16-128µg/mL. However, the lowest MIC for doxycycline also appears to be well beyond therapeutic capacity. Other than the inherently high MICs, one thing that stands out is tight distribution of MICs. Both tetracycline and doxycycline only span

4x 2-fold increases. Due to the high overall MICs found in both tetracycline and doxycycline no further work was carried out on the smallest subset, which grew >128µg/mL.

6.5.1 Discussion

LASARUS on a solid agar dilution method gives high MICs for both tetracycline and doxycycline. Examining the susceptibility literature, which has investigated tetracyclines efficacy against *Legionella* spp., shows key differences between methods of MIC determination. To some degree these were shown in Results Chapter 2 Concordance and presented in (Portal et al, 2021). This showed a lack of concordance with LASARUS and the gold standard (BMD in BYE-α) and gradient strips on, or agar antibiotic dilution in BCYE-α as per Figure 5.4.

For tetracycline, three reports have investigated MICs: two used gradient strips on BCYE-α and one used BMD. Natås *et al* using gradient strips reported MIC_{50} of 2µg/mL and an MIC_{90} of 8µg/mL (n=122). Sreenath *et al* 2019 using gradient strips reported an MIC_{50} of 1µg/mL and an MIC_{90} of 2µg/mL (n=46). Wilson *et al* 2018 using BMD, reported an MIC_{50} and MIC_{90} of 4µg/mL. (Wilson et al. 2018; Natås et al. 2019; Sreenath et al. 2019). These results are highly discrepant with the data generated by agar dilution in LASARUS for tetracycline above, where the MIC_{50} and MIC_{90} 128µg/mL.

For doxycycline there are more papers investigating MICs, likely due to its clinical significance. Of the 12 papers published since 1997 one has used BCYE-α agar

dilution and had an MIC_{50} of 8µg/mL and an MIC_{90} of 32µg/mL (n=32) (Marques and Piedade 1997). Nine papers have investigated doxycycline using gradient strip giving a total sample of (n=882) investigated isolates. These have an MIC_{50} range of between 0.032-4µg/mL and an MIC_{90} range of 0.5-8µg/mL (Rhomberg et al. 1994; Marques and Piedade 1997; Bruin et al. 2014; De Giglio et al. 2015; Koshkolda and Lück 2018; Torre et al. 2018; March et al. 2019; Sharaby et al. 2019; Sreenath et al. 2019). Two papers have used BMD: Vandewalle-Capo in 2017 showed an MIC_{50} of 1µg/mL and an MIC_{90} of 2µg/mL (n=109) and Xiong in 2016 showed MIC_{50} of 8µg/mL and an MIC_{90} of 8µg/mL (n=60) (Xiong et al. 2016; Vandewalle-Capo et al. 2017). These results are highly discrepant with the data generated by agar dilution in LASARUS for doxycycline, which I have presented above, where the MIC_{50} and MIC_{90} 32µg/mL.

This discordance was also found when I performed these three methods in unison on the same isolates as detailed in Figure 4.1. Even when performed on the same day, under the same conditions, LASARUS gave a consistent 2-fold higher MIC than BMD, and gradient strips gave a consistent 4-8 fold relative decreased MIC. If such variation is observed when tested in parallel, it is not surprising to find such variation in the literature. Furthermore, these readings below were taken at five days, whereas Wilson *et al*.'s BMD readings were taken at 48 hours, which may also explain their lower finding of an MIC_{50} of 4µg/mL, 4-fold lower than the BYE-α results in as through growth on a bacteriostatic antimicrobial.

Another possible explanation for this difference in results could have been an electrolyte content of the LASARUS, which may have acted as a chelating agent in our media thereby artificially elevating MICs as was proposed by (Isenman et al. 2018).

From a clinical perspective tetracyclines are rarely but occasionally used in the treatment of hospital and community acquired pneumonias, and from the literature tetracyclines appear effective in the treatment of *Legionella* infection in both human and animal models but it does set tetracycline resistance thresholds (Edelstein et al. 2003; Teh et al. 2012). This highlights the problems with *in vitro* testing not necessarily correlating with *in vivo* effect.

6.6 Chloramphenicol

6.6.1 Results

2,137 isolates were screened against chloramphenicol testing a range between ≤0.5µg/mL and 2µg/mL using the LASARUS agar dilution method described in the methods section 3.4.

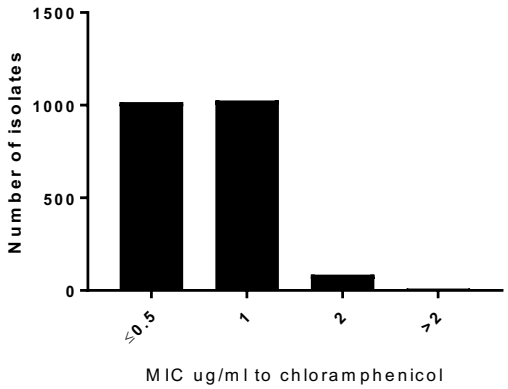


Figure 6.10 Chloramphenicol MIC determination for n=2,137 *Legionella* spp.
Initial experiments showing MIC isolates to chloramphenicol as per methods 3.4.

The distribution of MIC values in Figure 6.10 (n=2,137) could not be assessed for normality, as there were too few concentrations evaluated. However, it is still possible to assign an MIC_{50} of 1µg/mL (as less than half of the isolates were determined to be ≤0.5µg/mL) and an MIC_{90} of 1µg/mL. Of the isolates screened above, a few (n=32) grew above the maximum tested concentration of ≥2µg/mL, these were then subjected to further investigations. These 32 isolates were regrown, and MIC determined against a wider concentration range of chloramphenicol testing a range between 1 and 16µg/mL.

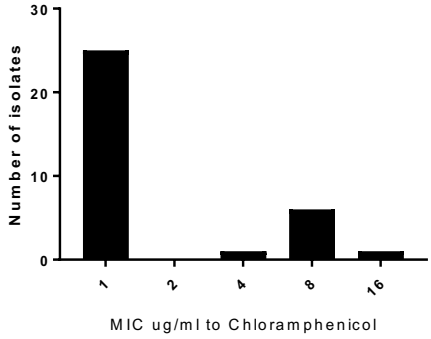


Figure 6.11 Chloramphenicol MIC of isolates with reduced susceptibility further determination for n=32 *Legionella* spp.
Follow up MIC isolates to chloramphenicol as per methods 3.4.

Of the isolates re-analysed, 24 had an MIC = 1µg/mL that represents a minor 2-fold variation common to biological replicates of dilution-based MIC experiments. However, a further eight isolates were confirmed to have an MIC that was in excess of the 2µg/mL, and a clear separation between these and the remaining isolates is apparent.

Of these further screening panel, one isolate had an MIC of 4µg/mL, which represented an 8-fold increased on the MIC_{50} of the cohort. Further investigations were not carried out on this isolate preferring to focus on the higher MIC strains. Six had an MIC of 8µg/mL and one isolate had an MIC of 16, which represents a 16-fold and a 32-fold increase from the MIC_{50} respectively. An individual colony was picked from the antibiotic negative control plate then sequenced by Illumina, MiSeq. Post sequencing, 51-09 and 20-70 were excluded at this stage as upon sequencing it was discovered they were contaminants (*Lactobacillus* spp.). The other six isolates, which demonstrated a significant resistance information regarding their ST, source isolation date and MIC to chloramphenicol are listed in Table 34.

Table 34 Additional information on isolates with chloramphenicol resistance

ID	ST	Clinical linkage	Source	Location	date isolates	Chloramphenicol MIC
45-36	37	X	Clinical	England	21/10/2016	16
49-03	74	Y	unknown	Unknown	11/09/2017	8
49-08	1012	K	Clinical	Greece	11/09/2017	8
49-09	42	K	Clinical	Greece	11/09/2017	8
51-09	47	L	Clinical	England	16/11/2017	8
52-38	256	M	Clinical	Unknown	15/01/2018	8

As all six isolates screened formed part of the recurring MDR cohort identified earlier.

They are further discussed in section 6.9.

6.6.2 Discussion

The first paper, which investigates the MIC of chloramphenicol, looks at six isolates (four from the Philadelphia outbreak 1976, one from the Flint Michigan and one from the Pontiac Michigan outbreak 1968). This work was carried out using an MHIH (Mueller-Hinton agar supplemented with 1% IsoVitaleX and 1% haemoglobin) and, showed a result of 0.5µg/mL for all which agrees with our screening (Thornsberry et al. 1978). An Edelstein review in 1995 highlighted a range of extracellular MICs between 0.5 and 1µg/mL, and stated that due to the increased efficiency of erythromycins and the side effect profile of chloramphenicol (Edelstein 1995). My data confirms that *Legionella* spp. remain susceptible to chloramphenicol, and agrees with Edelstein that with the availability of other antibiotics that lack the side effects of chloramphenicol and is not recommended for *Legionella*.

This work is one of the first to show the MIC of chloramphenicol against *Legionella* spp. and is by far the largest completed to date. In screening 2,139 isolates, an MIC_{50} of 1µg/mL and an MIC_{90} of 1µg/mL allows determination of a resistance isolate to be one that has an MIC of 4µg/mL or higher.

6.7 Azithromycin

6.7.1 Results

In total 2,185 isolates were screened against azithromycin with a testing a range between 0.008µg/mL and 0.25µg/mL on LASARUS.

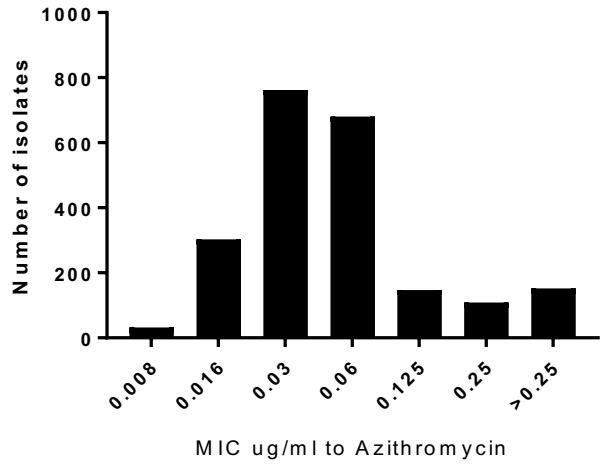


Figure 6.12 Azithromycin MIC determination for n=2185 *Legionella spp*

Initial experiments showing MIC isolates to azithromycin as per methods 3.4

The MIC_{50} for azithromycin was 0.03µg/mL and the MIC_{90} is 0.25µg/mL. This graph is of interest due to the non-Gaussian distribution profile of the results. This rising increase at the MIC >0.25µg/mL, represents a second overlapping distribution. All isolates within this group were re-assayed for MIC determination using a wider concentration of azithromycin of 0.06-1µg/mL.

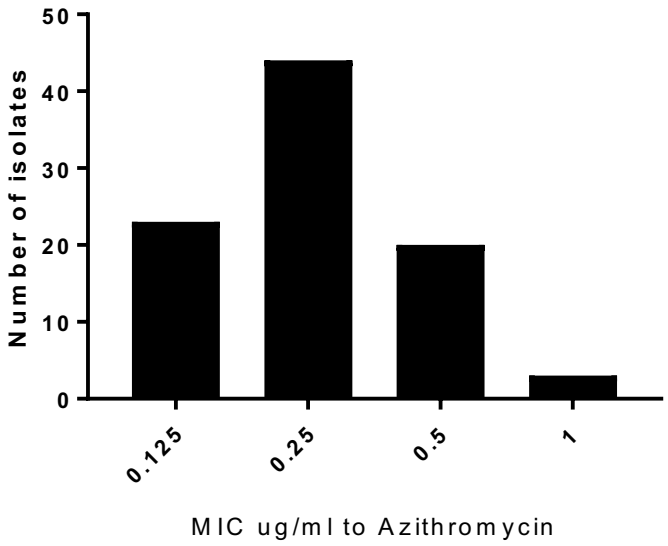


Figure 6.13 Azithromycin MIC determination for n=97 *Legionella spp.*
Follow up experiments showing MIC isolates to azithromycin as per methods 3.4.

Re-analysis, found that 23 isolates had an MIC of 0.125µg/mL, 45 isolates had an MIC of 0.25µg/mL (representing the common one or two dilution variances for biological replicates). Twenty isolates had an MIC of 0.5µg/mL and three isolates had an MIC of 1µg/mL, which would have been above the highest concentration examined in the large-scale screening. Considering overlapping bimodal, distributions of MICs for azithromycin, it is impossible to speculate where to set a theoretical threshold for *in vitro* resistance. While the distribution drops sharply for isolates that have an MIC <0.06µg/mL; approximately 15% of the cohort above that range (>300 isolates) and the MIC_{90} is 0.25µg/mL. Therefore, assigning a 95% cut off would be unreasonable. As such I have focussed further investigation on isolates >0.06µg/mL. Due to the significant interest in isolates with the highest MICs particularly the isolates with an MIC at 1µg/mL, these were the primary focus as they demonstrate the most significant decrease in susceptibility relative to the remainder of the cohort.

The subset of isolates selected for sequencing, to investigate resistance mechanisms are presented in Table 35. This included the three isolates that had an MIC of 1µg/mL and representative isolates with MICs of 0.125-0.5µg/mL. Isolates are identified through their anonymising code and the associated MIC are detailed in Table 35.

Table 35 Sequenced isolates with reduced sensitivity to azithromycin

ID	ST	Source	Source	Date Isolated	MIC	Resistance genes	Additional Information
53-25	1	Environmental	Unknown	28/03/2018	1	*lpeAB*	
24-36	1	Clinical	England	25/08/2011	1	*lpeAB*	Fatal, ITU
52-38	256	Clinical	Unknown	15/01/2018	0.5		MDR
44-80	1	Environmental	Wales	06/10/2016	0.25	*lpeAB*	
34-29	1	Environmental	Unknown	11/06/2014	0.25	*lpeAB*	
44-74	1	Environmental	Wales	06/10/2016	0.25	*lpeAB*	
44-76	1	Environmental	Wales	06/10/2016	0.25	*lpeAB*	
49-07	23	Clinical	Greece	11/09/2017	0.25		MDR
49-03	74	Unknown	Unknown	11/09/2017	0.25		MDR
49-08	1012	Clinical	Greece	11/09/2017	0.25		MDR
41-24	1717	Clinical	Wales	30/12/2015	0.25		
45-02	1	Clinical	Wales	07/10/2016	0.125	*lpeAB*	
45-01	1	Clinical	Wales	07/10/2016	0.125	*lpeAB*	

As the isolates were selected based on elevated MICs as the sole criteria, comparison of the metadata in Table 35 identified some interesting findings. The 13 strains had isolation dates ranging from 2011 to 2018, those with identical collection dates had identical MIC values. They were a mix of both clinical and environmental isolates (where the data was known) and at least two isolates represent travel-associated LD as their source was documented as “Greece” these are likely to be the same pick from a single patient. Where recorded (three were listed as unknown) they were largely collected in Wales, with one from England. No further data was available for these strains. MLST analysis of the WGS data revealed that 8/13 (61.5%) strains were *Legionella pneumophila* Sg 1 ST 1 and all that carried the *lpeAB* resistance gene.

Presence of *lpeAB* was found for isolates with MICs at both ends of the 0.125-1µg/mL range. The highest MIC of 1µg/mL isolates (53-25 and 24-36) were both ST1 containing, the efflux pump encoding gene *lpeAB,* the most likely cause of the resistance.

However, 53-25 and 24-36 were both 4-fold higher than the rest of the *lpeAB* containing strains, interspersed with isolates lacking *lpeAB* this warrented higher resolution genomic interrogation. Therefore, genomic DNA was additionally submitted for MinION sequencing of these isolates to generate a whole sealed complete genome with full characterisation of sealed associated plasmids.

An investigation into *Legionella* isolates with elevated MIC possessing the *lpeAB* gene was further carried out to investigate the up- stream pathways and promotor region. The aim of this was to find a SNP within the promotor regions of the higher MIC isolates compared to the lower MIC *lpeAB* isolates. This could then have been correlated to patient history to determine if the patient had been treated with azithromycin prior to sample collection, given the literature for *in vitro* induction of promoter mutation with increased *lpeAB* expression as mentioned by (Massip et al. 2017). Characterisation of *lpeAB* gene promotor region in *Legionella pneumophila* strain Paris showed that the promotor region was 202bp long. Aligning this promoter and gene prototype to five isolates of interest with ranging MICs from 0.125-1µg/mL, showed 100% consensus with Paris as can be seen in the Figure 6.14 generated from Geneious prime. The sole difference is that isolate 34-29 lacked the cold-shock protein but this isolate was not a highly resistant strain (0.25µg/mL).

Figure 6.14 Promotor regions of *lpeAB* mapped to *Legionella pneumophila Paris.*

This figure shows the 100% concordance within the 202bp promotor region of the *lpeAB* gene. with 7 of the strains with reduced azithromycin susceptibility.

Interestingly Paris, which is the type strain for *Legionella* possessing *lpeAB* has an ICE insertion just upstream from the *lpeAB* gene, splitting the SidH gene in two. When comparing the promotor region of *lpeAB* with a strain which doesn't contain *lpeAB*, such as the Philadelphia strain, I can see where the gene would have inserted and the homology of the promotor region for the first 196~bp, followed by another 150~bp of non-coding sequence as seen in Figure 6.15.

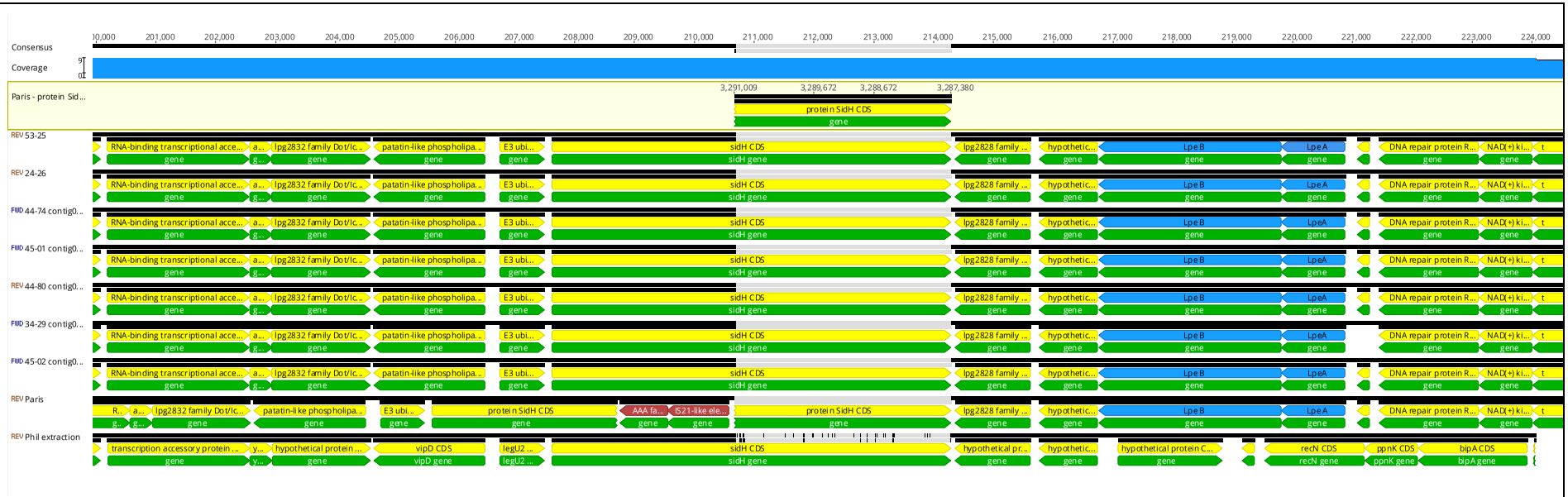


Figure 6.15 An alignment of the SidH gene showing an IS21$_{\text{-like}}$ element in *Legionella pneumophila* Paris

This diagram from Genious prime shows 7 azithromycin resistant strains mapped to the SidH gene of *Legionella pneumophila* Paris. With LpeA+B genes in blue and the insertion element an IS21$_{\text{-like}}$ sequence inserted into the middle of the SidH gene in Paris.

Figure 6.16 LpeA and B insertion site

This figure shows the point at which *lpeA* and *lpeB* would have been inserted, this shows a 338bp region of non-coding DNA the first 190bp are identical to the promotor region of *lpeAB* in the above tested isolates.

ST1 isolates all contained the *lpeAB* gene. Presence of *lpeAB* has been shown in a few other sequence types as described, ST1 and ST1$_{\text{-like}}$ (ST6 and 177) as well as in a separate clade of two isolates (ST701 and 259) (Vandewalle-Capo et al. 2017). Natas *et al* showed that all ST1 (n=18) isolates they screened had reduced susceptibility to azithromycin, and all contained *lpeAB*. They also showed three other isolates with reduced azithromycin susceptibility; one L.p Sg4 isolate and two L.p Sg5, ST1973 and ST1328 respectively. These three isolates all possessed an *lpeAB* gene homolog with ~90% sequence identity (Natås et al. 2019). Jia *et al* showed *lpeAB* carriage in four strains within L.p Sg1 but not ST1. These were ST144, ST150, ST154 and ST629 and all map closely to ST1, but all were determined by SBT Sanger sequencing (Jia et al. 2019). This is prone to misalignment and error and as such further investigations should be carried out to confirm if these are in fact true non-ST1 isolates (Potapov and Ong 2017).

I found, five isolates that were not ST1 and did not carry the *lpeAB* gene; therefore, an alternative mechanism of resistance was investigated. All sequenced isolates were screened through ResFinder and none were found to contain other resistance genes responsible for macrolide resistance. The most up to date database (12/2021) was utilised, which included the most likely characterised genes including *ermA*, *ermB*, *ermC*, *ermF*, *mphA*, *ereA* and *ereB*; nor were any other less common resistance genes reported in the literature found. Of course, these databases are limited to known genes and it is possible that these 5 isolates contain a novel efflux pump, as a limitation of this method of interrogation.

In further investigations as to the cause of the varying degrees of resistance found in isolates containing *lpeAB*, which lacked other known resistance genes, I investigated potential resistance-mediating mutations in the 23S rRNA operons. All these isolates belonged to *Legionella pneumophila* which contains three rRNA operons.

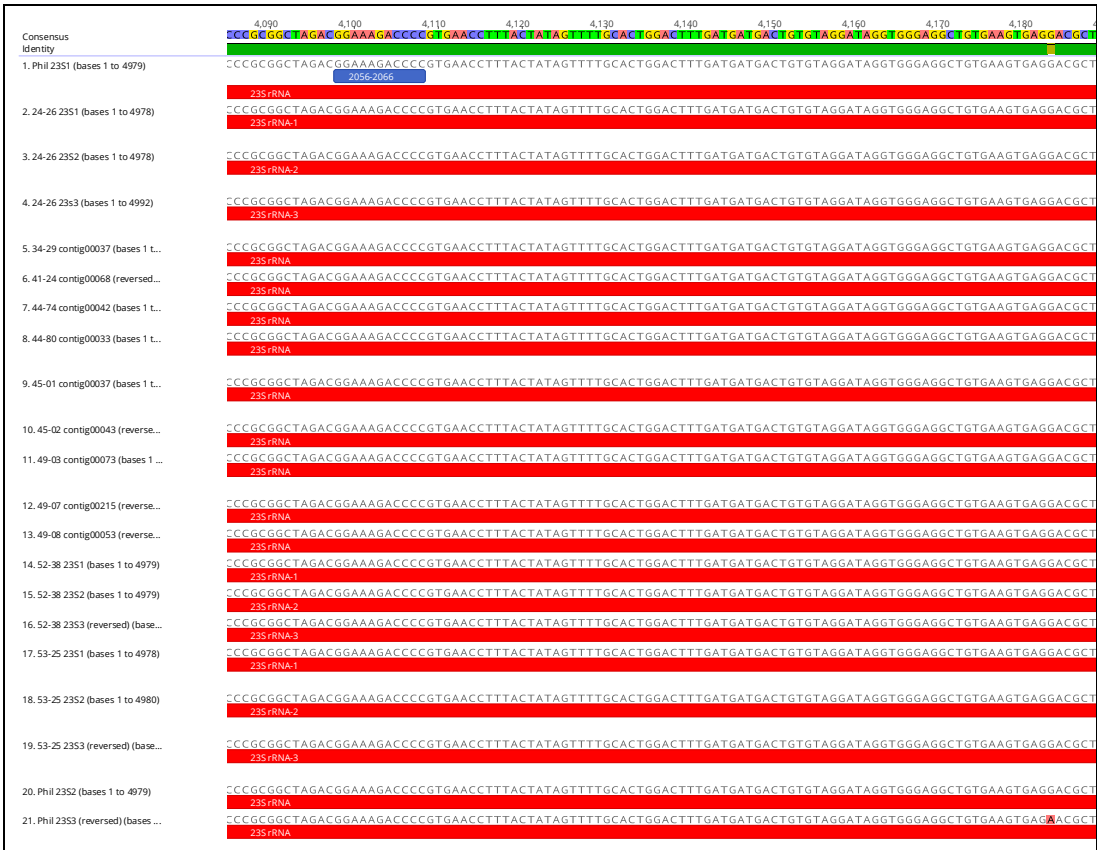


Figure 6.17 23S RNA for the azithromycin resistant strains

This figure shows the 23S RNAs for the isolates tested with reduced azithromycin susceptibility as well as *Legionella pneumophila* Philadelphia, highlighting no changes in the 2056-2066 region in the 23S.

Resistance to azithromycin in these strains ranged from 0.125-1µg/mL and no ribosomal mutations were found Figure 6.17. The *in vitro* work carried out by showed that a single mutation of A2056G induced an MIC of 32µg/mL. They also reported occasional resistance-mediating mutations in the parallel region C2611G (Descours et al. 2017). However, none were found in our high azithromycin samples.

One isolate in this cohort stood out as being of particular interest. The ST1717 strain isolated from a Welsh clinical sample in 2015 was one of seven other isolates collected on the same date from the same patient (presumably processed as individual colonies): all with the same ST and MIC to azithromycin of 0.03µg/mL. Whereas 41-24 had an MIC of 0.25µg/mL (8-fold higher and a result confirmed on repeated analysis). This isolate did not contain *lpeAB* nor was its part of the MDR collection of isolates.

6.7.2 Discussion

Screening of 2,185 *Legionella* isolates from the UKHSA archive to azithromycin determined an overall MIC_{50} of 0.03µg/mL and an MIC_{90} of 0.25µg/mL.

Comparing this to the literature using gradient strip on BCYE-α of which there are 11 papers, shows an MIC_{50} range of 0.047-0.38µg/mL and an MIC_{90} range of 0.19-1µg/mL. The MIC_{50} which if converted to the closest log form number would be between 0.06-0.5 represents a 2-16fold increase on our reported results. The MIC_{90} if similarly converted shows an MIC_{90} range of 0.25-1µg/mL representing a static to 4-fold increase on our results further highlighting conclusions in results chapter 2, of elevated MIC results when testing on BCYE-α. When comparing with BMD the MIC range between 0.016 and 4µg/mL as found by (Xiong et al. 2016; Vandewalle-Capo et al. 2017) which more closely matched my findings.

There were several important findings that arose from interrogation of the strains that were found to have elevated azithromycin MICs:

1) Only 8/13 of these isolates carried the *lpeAB* gene and they were not concentrated at the highest MIC concentrations.

2) ST1 universally carries the *lpeAB* geneand other STs which do carry *lpeAB* cluster around clinically similar STs to ST1, as published by (Vandewalle-Capo et al. 2017; Natås et al. 2019).

3) No mutations in the promoter region of the *lpeAB* gene were found to be associated with the strains with higher or lower MICs of 1µg/mL. The *lpeAB* gene was found on the somatic genome located between the cold shock protein (LPP_RS14520 locus tag) and hypothetical protein (LPP_RS14535 locus tag) as found in the prototype ST1 *lpeAB*-positive Paris strain (NC_006368 GenBank submission).

4) No previously identified antimicrobial resistance gene or macrolide resistance mediating mutation was found in strains with elevated MICs but were *lpeAB* negative. The mechanism of elevated azithromycin MIC is still unknown for these isolates and will require comparison to the closest genomically related susceptible strain with advanced genomic analysis to attempt to determine this mechanism. However, there have been no previous reports of clinical or environmental isolates with elevated macrolide without the concurrent presence of the gene *lpeAB*. The isolates 53-25 and 24-36 represent the first identification of "macrolide resistant" *Legionella pneumophila*; however, this should be viewed in the context that these MICs are not likely to escape monotherapy treatment with azithromycin.

L. pneumophila Sg 1 ST1 is the most prevalent ST in patients in the USA, the UK and is one of the most prevalent STs globally. It is often found in environmental samples and ST1 is positively associated with worsened disease outcome (Ginevra et al. 2020). They all carry the *lpeAB* gene and the low efficacy of this gene in elevating macrolide MIC above a clinically relevant level means this gene cannot be used as a marker of macrolide resistance and to some degree should probably be consigned to history as unimportant with regards to clinical relevance (as it is unlikely to be responsible for treatment failures). Now that guidelines for AMR screening have been proposed hopefully systematic investigation will determine the true rate of macrolide resistance in *Legionella pneumophila* (Portal et al. 2021b).

6.8 Rifampicin

6.8.1 Results

2,050 isolates were screened against rifampicin testing a range between 0.002µg/mL and 0.008µg/mL.

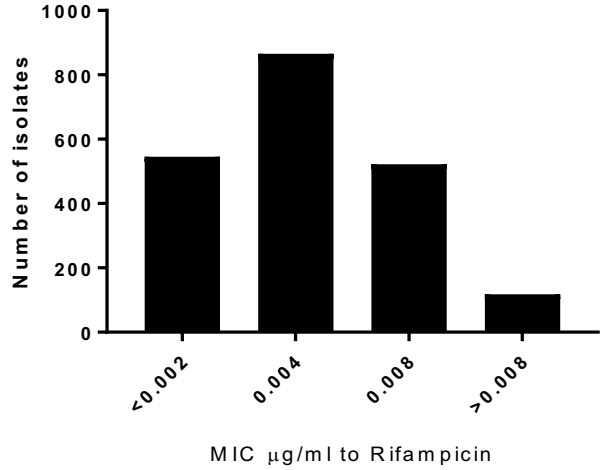


Figure 6.18 Rifampicin MIC determination for n=2,050 *Legionella spp.*
Initial experiments showing MIC isolates to rifampicin as per methods 3.4.

The distribution of MIC values in Figure 6.18 above (n=2,050) has the appearance of a Gaussian (normal) distribution. These isolates yielded an overall MIC_{50} of 0.004µg/mL and an MIC_{90} of 0.008µg/mL.

However, all isolates with an MIC >0.008µg/mL were subjected to further investigations. These seventy-five isolates where regrown and re-analysed against a wider concentration range of rifampicin (0.004-1µg/mL).

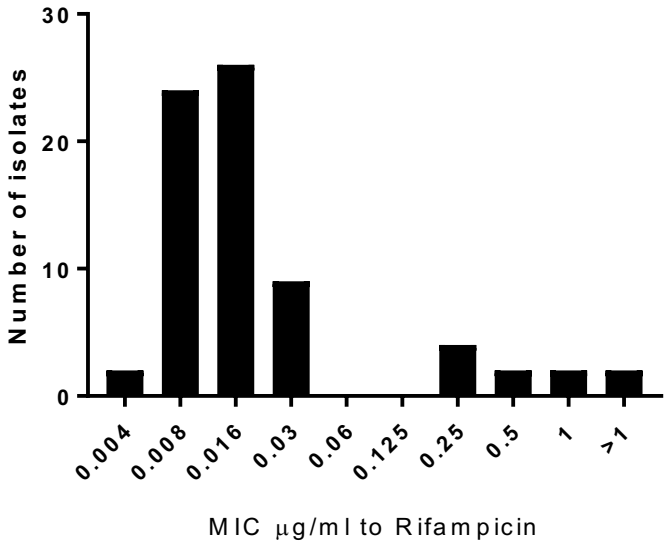


Figure 6.19 Rifampicin MIC determination for n=75 *Legionella spp*
Follow experiment showing MIC isolates to rifampicin as per methods 3.4.

Of the 75 isolates re-analysed, two isolates were found to have an MIC=0.004µg/mL and 24 were found to have an MIC=0.008µg/mL, which represented a minor 2-fold variation that is common to biological replicates of dilution-based MIC experiments. However, a further 49 isolates were confirmed to have an MIC that was in excess of the 0.008µg/mL used in the initial triage experiments. These minor number of higher MIC would not increase the MIC_{90} of 0.008µg/mL for rifampicin; however, where to set a theoretical threshold for in vitro resistance is not immediately apparent. Certainly, there is a clear break between isolates that have an MIC >0.06µg/mL, that must be considered as demonstrating a significant decrease in susceptibility relative to the remainder of the cohort. However, isolates with an MIC= 0.03-0.06µg/mL fall at the boundary of the 95% cut-off, assuming a Gaussian distribution. While no further analysis was carried out on the 35 isolates which had an MIC of between 0.016 and 0.03µg/mL, they may represent variants of reduced susceptibility and had time

permitted they would have been re-analysed further to define the stability of these MIC results.

There were seven isolates which demonstrated a significant relative resistance were sequenced. Their strain identifiers are shown in Table 36.

The first unexpected result was that 3/7 isolates which had the macroscopic appearance and growth characteristics consistent with *Legionella* were in fact other bacterial species. Isolates ID-20-70 (MIC=>1), 51-09 (MIC=0.5), and 40-19 (MIC=1) were contaminants, (this would have been identified using a MALDI-ToF on colonies routinely employed in the earlier experiments, however due to the COVID-19 pandemic, this equipment was inaccessible and therefore these were not further investigated.

The other isolates 45-36, 49-07, 49-03 and 38-52 were confirmed as *L. pneumophila* and examined for their resistance mechanism.

Table 36 Showing isolates with rifampicin resistance

ID	MIC	initial sequencing results	source	ST	Date of isolation
51-09	0.5	*Lactobacillus paracasei*			
40-19	1	*Lactobacillus paracasei*			
20-70	>1	*Methylobacterium spp.*			
45-36	0.25	*L. pneumophila*	England, Clinical	37	2016
49-07	0.5	*L. pneumophila*	Greece Clinical	23	2017
49-03	1	*L. pneumophila*	Unknown Clinical	74	2017
52-38	>1	*L. pneumophila*	Unknown Clinical	256	2018
24-36	1	*L. pneumophila*	England Clinical	1	2011

Resistance as determined by ResFinder failed to identify any potential mechanisms of resistance for these isolates. However, this resistance database does not show somatic mutations (aka SNPs), which may cause resistance, so this was further examined by gene alignment.

Rifampicin resistance in other bacterial species are well characterised and are often mediated through RpoB mutations. Amino acid polymorphisms within key structural regions cause conformational changes preventing rifampicin from inactivating the subunit; therefore, this was investigated as the next most likely cause of resistance. The non-synonymous SNPs were investigated using GeneiousPrime software and aligned against sequences from a selection of non-rifampicin resistant *Legionella pneumophila* isolates And are shown in Table 37 shows. The resistant isolates commonly contained nonsynonymous SNPs. However no polymorphisms were unique to resistant strains (38-52, 49-03 and 49-07), all of these were also present in the rifampicin susceptible isolates and therefore could not be responsible for the observed phenotype.

Table 37 Highlights non-synonymous SNPs within the RpoB subunit

This table highlights non synonymous SNPs comparing rifampicin resistant and susceptible isolates and shows that

							MIC->1				MIC-1	MIC-0.5				
30-23	**30-59**	**41-24**	**44-45**	**44-74**	**21-09**	**24-36**	**38-52**	**43-07**	**45-24**	**45-61**	**49-03**	**49-07**	**49-08**	**49-13**	**52-38**	**53-25**
										D49N						
				T220S		T220S			T220S			**T220S**	T220S			T220S
S235N					S235N									S235N		
H242C	H242R	H242R	H242R	H242R	H242C	H242R	**H242R**		H242R	H242R	**H242R**	**H242R**	H242R	H242C	H242R	H242R
										Q291L						
N342S					N342S	N342S				N342S				N342S		
										A640E						
										D1015E						
		E966D														
		I1035V		I1035V		I1035V	**I1035V**		I1035V	I1035V		**I1035V**	I1035V		I1035V	I1035V
				L1163R		L1163R	**L1163R**		L1163R			**L1163R**	L1163R		L1163R	L1163R
										L1170R						
										T1172F						
										R1203L						
	A1204S		A1204S								**A1204S**					
		T1217S														
						P1240T			P1240T							P1240T

These five isolates are the first reported isolates to contain naturally occurring resistance to rifampicin and as such determination of the underlying mechanism of resistance is of importance regardless of the overall MIC or its clinical relevance.

Table 38 Addition information on resistant *Legionella* isolates.

ID	Location	Species	ST	Additional information
52-38	Patient	*L. pneumophila*	47	Travel UK, survived
49-03	Patient	*L. pneumophila*	74	
49-07	Patient	*L. pneumophila*	23	Travel abroad (Greece), survived
45-36	Patient	*L. pneumophila*	37	
24-36	Patient	*L. Pneumophila*	1	UK

Of these isolates all bar 24-36 are resistant to multiple antibiotics and as such are further discussed in 6.9 Results of re-evaluation of strains with multi-drug resistance.

6.8.2 Discussion

The MIC_{50} and MIC_{90} of rifampicin are low ensuring that isolates at the higher end of the MIC curve are still well within the therapeutic ranges of 8-24µg/mL (Peloquin 2002; Aït Moussa et al. 2016). Comparison of these results to previously published data is problematic, due to methodological variations, where the presence of activated charcoal in the solid medium absorbs antibiotics to varying degrees (Portal et al. 2021a). However, comparison to a report utilising BMD showed high concordance to our findings with similar MIC_{50} and MIC_{90} for *Legionella pneumophila* (Gómez-Lus et al. 2001) All isolates tested on BCYE-α agar dilution gave MIC_{50}between 2-32 times higher than our findings and for other reports using BMD methods (Liebers et al. 1989; Marques and Piedade 1997; Ruckdeschel and Dalhoff 1999; Tsakris et al. 1999). The elevated MIC determination appears to be less pronounced compared to the BCYE-α gradient (E-strip) methods, which on average show an MIC_{50} and MIC_{90} 4-fold higher than our study (Bruin et al. 2012; Torre et al. 2018; Natås et al. 2019). Comparison of these methods is discussed in more detail in Chapter 4 and recent publication (Portal et al. 2021b), our results concur with the above-mentioned studies that highlights rifampicin's *in vitro* bactericidal efficacy in *Legionella* spp. The clinical utility of rifampicin in the treatment of LD is less clear, as outlined in clinical trials, and a more robust double blinded trial would be necessary to prove the benefit of a combination therapy over therapeutic toxicity risks as well as the risk of driving these isolates towards resistance.

When analysing mutations which confer rifampicin resistance within the RpoB protein, these mutations cluster in three regions: aa507-533, aa563-572 and at aa687

all within the centre of the RpoB gene, these clusters have been named as the Rifampicin Resistance Determining Region (RRDR) (Xu et al. 2005; Goldstein 2014).

Mutations suspected to confer resistance have been reported outside the RRDR, but are rare (Goldstein 2014). These mutations are usually single point mutations, but codon deletions and insertions have also been reported. By comparing the RpoB sequence from isolates that had greatly increased rifampicin MICs to those that were much more sensitive to rifampicin, I could not identify any amino acid changes that were not present in both phenotypes. More importantly, no isolates were found to have any RpoB mutations between aa342 and 640. RNAp mutations with reduced rifampicin susceptibility have been reported in Legionellaceae*:* an investigation into rifampicin resistance by Neilson *et al* characterised six rifampicin-resistant *Legionella* that had mutations around the Beta-subunit considered in *M. tuberculosis, E coli* and other species to be a key site for rifampicin mutations (Nielsen. et al. 2000). These mutations fall within the aforementioned RRDR mutations highlighted in Figure 6.20. A screen of all my resistant isolates failed to highlight any of these mutations, nor any in the key aa687 region of the beta-subunit.

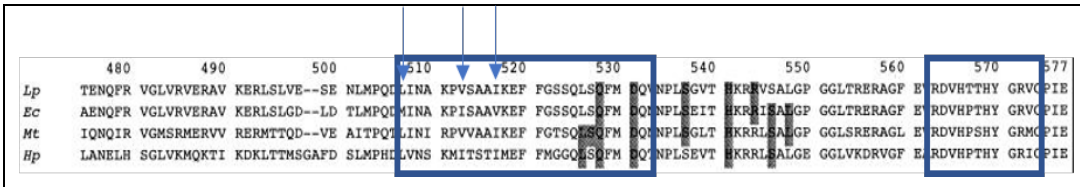


Figure 6.20 Alignment of the Beta subunit of the RNA polymerase
Alignment of partial β-subunit sequences of the RNA polymerase genes of *L. pneumophila*, *E coli*, *M tuberculosis*. Using conventional *E. coli* numbering. The blue boxes represent the RRDR, with the blue arrows denoting amino acid changes compared to the E. coli stains within the RDRR. Modified from: (Nielsen. et al. 2000)

Non-mutation mediated mechanisms of rifampicin resistance have also been reported by Louw *et al,* the presence of efflux pumps has been reported for low level rifampicin

resistance in mycobacteria, but as of yet none have been found in Legionellaceae (Louw et al. 2009). As all of my sequences were interrogated against resistance gene databases, and these genes are included in the ResFinder database, known efflux pumps can be excluded as causing the elevated rifampicin MICs in this study. However, the potential for uncharacterised novel efflux pumps as the mechanism of reduced susceptibility to rifampicin cannot be ruled out.

Only five out of 2,050 isolates had an MIC greater than MIC_{90} were identified, no obvious mechanism of resistance in RpoB mutations nor efflux pump presence were found.

6.9 Results of re-evaluation of strains with multi-drug resistance

Where isolates for each antibiotic were found to have suspected resistance, the corresponding isolate was sequenced from the antibiotic free control plate in an effort to determine the underlying resistance mechanisms. All of these sequences, and a subset of susceptible strains for comparison, were analysed to determine relatedness. The Figure 6.21 shows the core genome phylogeny to determine any relationship within the resistant isolates. For ease of analysis additional data has also been included alongside of the phylogenetic tree: the antibiotics for which they had elevated MICs, their ST, their isolation date, and their source (environmental or clinical), and their country of origin.

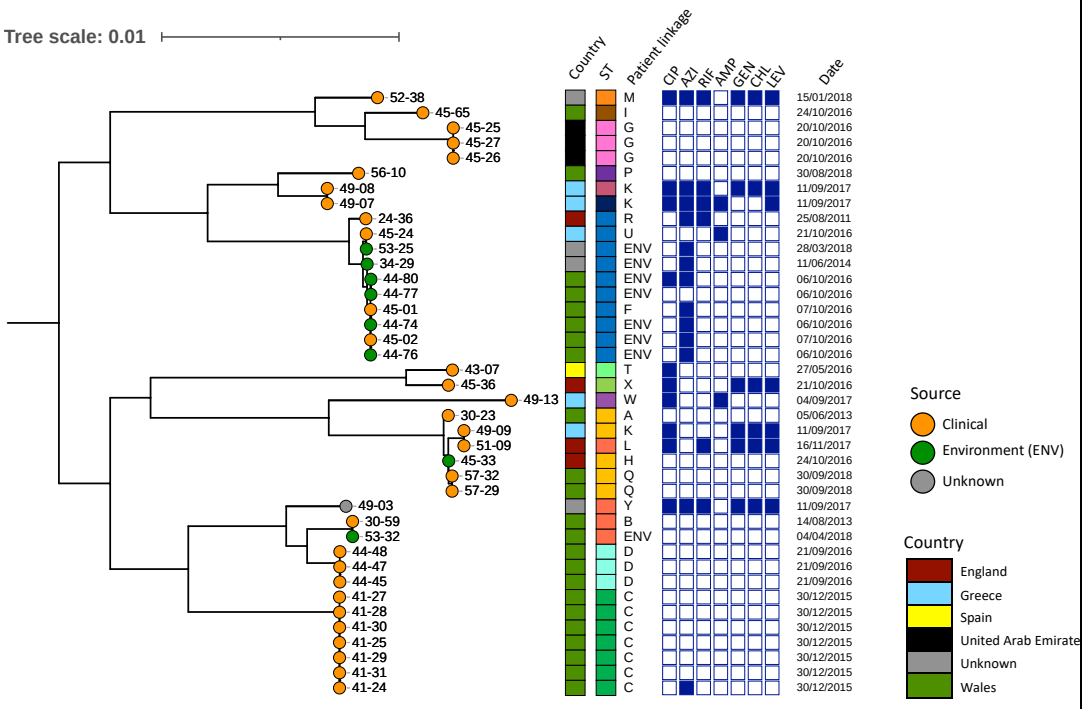


Figure 6.21 A Detailed core genome characterization of 40 *Legionella pneumophila*

A Detailed core genome characterization of 40 *Legionella pneumophila* isolates analysed in this thesis. The colours in the circles on the left indicate source (environmental or clinical), followed by the coloured boxes denoting country of origin, and sequence type. Where the sample represents multiple picks from the same patient this has been indicated in patient linkage. The blue boxes represent resistance to an antibiotic (CIP- ciprofloxacin, AZI-azithromycin, RIF-rifampicin, AMP-ampicillin, GEN-gentamicin, CHL-chloramphenicol, LEV-levofloxacin). The date displayed is the date of original isolation.

Immediately apparent are the MDR isolates (resistant to 3+ antibiotics), these are isolates 52-38, 56-10, 49-08, 54-36, 49-09, 51-09 and 57-29. These isolates are further investigated and shown in Table 40. There also appears to be an apparent bias towards clinical and Welsh isolates. The increased numbers of clinical isolates can be explained as significantly more clinical isolates were screened within this project. The apparent Welsh bias can be explained as funding for in depth examination of sequences for this subset was obtained and run in parallel during my studentship, and they are included as non-resistant sequences, to give some additional context to the resistant strains.

This is particularly apparent with regards to the clonal cluster of isolates surrounding isolate 41-24; where six separate colonies (41-24 to 41-30) from the same patient were examined and sequenced, resulting in the observed phylogenetic clustering, despite not sharing resistance to azithromycin observed for colony 41-24. Also of interest are the two ST1s; 45-24 and 44-77, which were not azithromycin resistant even though they contained the *lpeAB* gene further emphasising the small effect that *lpeAB* has on MICs.

The potential presence of MDR *L. pneumophila* clinical isolates was concerning and warranted targeted, in-depth interrogation. The isolates of concern were 52-38, 49-03, 49-08, 51-09, 49-07, 49-09, 45-36. Table 39 highlights their ST clinical linkage origin and isolation date. There were other isolates that had initially flagged as resistant, but on retesting they proved to be either contaminants or failed to exhibit repeated resistance (+2-fold serial dilution).

Table 39 background information on MDR isolates

ID	ST	Clinical Linkage	Source	Location	Date Isolates
52-38	256	M	Clinical	Unknown	15/01/2018
49-03	74	Y	Unknown	Unknown	11/09/2017
49-08	1012	K	Clinical	Greece	11/09/2017
51-09	47	L	Clinical	England	16/11/2017
49-07	23	K	Clinical	Greece	11/09/2017
49-09	42	K	Clinical	Greece	11/09/2017
45-36	37	X	Clinical	England	21/10/2016

Table 39 shows all these isolates clustering around a ~2-year period between the end of 2016 and the beginning of 2018, with four isolates with the same isolation date. They were from a diverse origin spanning England, Greece and two isolates with no recorded origin. Of the seven isolates tested, three were linked to a single patient (K) showing three distinct and separate STs. It is important to re-iterate at this point, that sequence analysis for these isolates to this point, were all derived antibiotic negative

control growth plate, and kmer analysis of the sequences confirmed they were all *Legionella pneumophila*. Table 40 shows the collective MICs for the antibiotics investigated of these isolates: three of which were resistant to six antibiotics, three were resistant to five, and one was resistant four antibiotics.

Table 40 A table of MICs of resistant *Legionella pneumophila*

ID	Ciprofloxacin	Azithromycin	Rifampicin	Ampicillin	Gentamicin	Chloramphenicol	Levofloxacin	Number resistance
52-38	**>2**	**0.5**	**0.5**	32	**4**	**8**	**>2**	6
49-03	**2**	**0.25**	**1**	16	**4**	**8**	**2**	6
49-08	**1**	**0.25**	**0.5**	8	**4**	**8**	**1**	6
51-09	**2**	**0.125**	**0.5**	16	**4**	**8**	**2**	5
49-07	**1**	**0.25**	**0.5**	16	**2**	**4**	**1**	5
49-09	**2**	0.125	0.004	8	**4**	**8**	**1**	4
45-36	**1**	>0.06	>0.008	>16	**16**	**16**	**1**	4

Bold= resistant

Given the lack of evidence for resistance mechanisms, these seven isolates were re-grown and re-analysed for susceptibility to the antibiotics. In all cases, high MICs were observed as previously recorded. To avoid having the expense and delay required for resequencing, qPCR was used to confirm *L. pneumophila* presence. All of the MDR isolates and five of the few *lpeAB*-positive azithromycin resistant isolates from Figure 6.21 were screened. As per 3.12 qPCR.

Table 41 showing qPCR Cq for high MIC samples

ID	Cq	CIP	AZI	RIF	AMP	GEN	CHL	LEV	#
49-03	17.02	1	1	1		1	1	1	6
49-08	16.4	1	1	1		1	1	1	6
52-38	28.5	1	1	1		1	1	1	6
49-07	15.48	1	1	1	1			1	5
51-09	16.81	1		1		1	1	1	5
45-36	20.47	1				1	1	1	4
49-09	15.43	1				1	1	1	4
44-80	17.16	1	1						2
45-61	N/A		1		1				2
49-13	17.24		1		1				2
34-29	17.1		1						1
41-24	15.14		1						1
43-07	15.31	1							1
44-74	15.41		1						1
44-76	17.18		1						1
45-24	18.72				1				1
53-25	14.92		1						1
Paris	28.43								0
Philadelphia	14.72								0

This table shows the *Legionella* qPCR of the *mip* gene reported as a Cq value. Isolates were picked at the lowest tested concentration of an agar dilution MIC experiment. Resistance to antibiotics is recorded as "1", with the total number of antibiotics with resistance tallied in the far-right column. MDR isolates are highlighted in yellow.

Table 41 confirms all isolates (bar one) contained *Legionella*. Examination of available sequence from isolate 45-61, which failed to be detected, identified a single base pair mutation present in the forward primer, which was not observed for the other sequences, and may be responsible for the failure.

However, despite the apparent uniformity of growth for the MDR isolates across the increasing concentrations of antibiotics, a discrepancy was observed for qPCR results from colonies taken from growth on the LASARUS plates with the highest isolate growth (Table 42).

Table 42 comparison of Cq for low and high MIC picks of the sample inoculation, Paris and Philadelphia as *Legionella* controls.

ID	Low MIC	High MIC
45-36	20.47	N/A
49-03	17.02	39.56
49-08	16.4	31.32
51-09	16.81	N/A
52-38	28.5	32.74
Paris	28.43	-
Philadelphia	14.72	-

Comparing the *L. pneumophila* bacterial load from the low and high antibiotic concentration plates showed that either *L. pneumophila* was not present (isolates 51-09 and 45-36) (Cq=NA), or almost barely present (Cq=31-39).

Isolates 49-03, 52-38, and 49-08 contained high levels of the *mip* gene (Cq=<16) were present, indicating the remaining growth was composed mostly of some co-contaminant with similar slow growth characteristics. Growth from these high concentration plates were prepared for whole genome sequencing and kmer analysis identified the resistant growth in these isolates to be either *Lacticaseibacillus* spp. or *Pediococcus acidilactici* (Table 43).

Table 43 Table of Cq and ID of contaminants

ID	**Cq**	**contaminate ID**
45-36 hMIC	NA	*Lacticaseibacillus rhamnosus*
49-03 hMIC	39.56	*Lacticaseibacillus paracasei subsp. paracasei*
49-08 hMIC	31.32	*Lacticaseibacillus paracasei*
51-09 hMIC	NA	*Lacticaseibacillus paracasei*
52-38 hMIC	32.74	*Pediococcus acidilactici*

This finding was disappointing, as the growth of these species, which were outcompeted by *L. pneumophila* (as confirmed by the qPCR results) in the absence of antibiotics, gave the false impression of MDR *Legionella* isolates.

Core-genome phylogenetic analysis of these isolates was carried out to determine if they were clonal and an indication of a contaminating source in the laboratory (Figure 6.22).

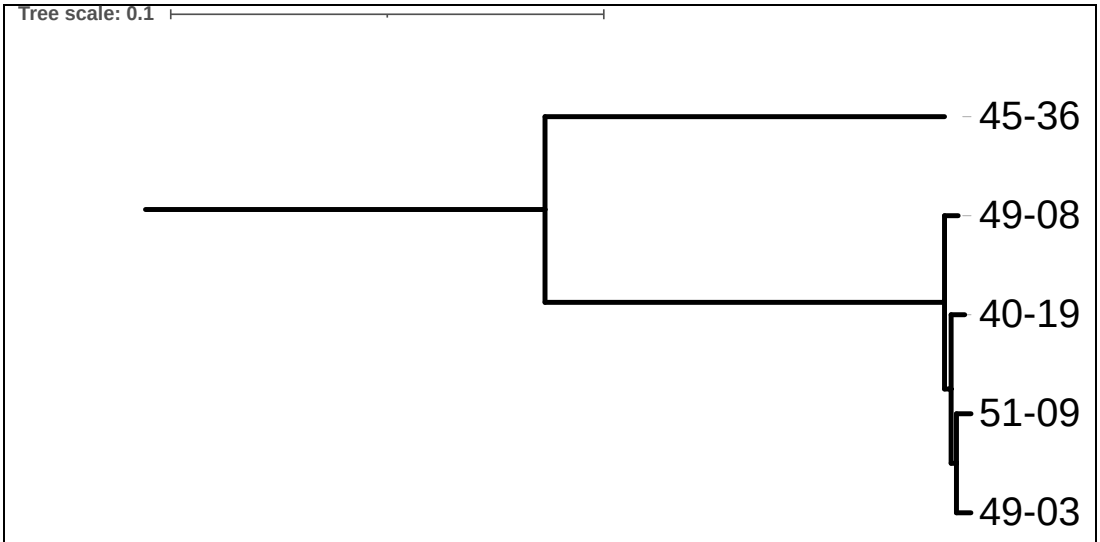


Figure 6.22 A core genome maximum alignment tree of five MDR contaminates

As this tree incorporate multiple species only 362 core genes have been included (from 6,438). The scale indicates that, a length 0.1= 10% difference between the genomes.

Figure 6.22 shows as expected the dissimilarity of 45-36 a *Lacticaseibacillus rhamnosus* from the rest of the cohort. However interestingly the remaining four isolates all *Lacticaseibacillus paracasei* were not clonal (there are 26,510 single nucleotide polymorphisms, 384 insertions and 387 deletions comparing 49-03 to 49-08), and therefore it can be concluded that this does not represent a single source of recent contamination. Had they been more closely related; it could have indicated contamination occurred at a source where the isolates were being processed for archiving, recovery from frozen archives, or preparation for AST (i.e. water bath or air-conditioning unit in the laboratory). If this had been random contamination at the point of screening, the contaminants would have been evenly mixed throughout the cohort of over 2,000 isolates screened. It is also notable that the contamination clusters

within archived isolates only spanning a two-year period: two of these isolates were isolated on the same day and an addition one 60 days later. However, the diversity of the genomic analysis still indicates a single source is not likely to be the source of contamination, either at Cardiff or at the UKHSA, and it is difficult to suggest a course of action for identifying where the *Lacticaseibacillus* spp. came from as the recent samples no longer have this contaminant.

Given the visual similarity of this organism to *Legionella* spp. When growing on LASARUS, incorporation of a selective agent that removes these contaminants and pinpoints *Legionella* chromogenically without affecting *Legionella* growth are essential.

6.10 Summarising AMR results

MIC on ~2100 isolates (between 2208-536) were determined and are summarised in Table 44.

Table 44 Overall MIC_{50}, MIC_{90} and range for all antibiotics tested

Antibiotic	MIC_{50}	MIC_{90}	Range	Number
Gentamicin	0.25	0.25	0.03-1	2,156
Ampicillin	8	32	1->128	2,175
Ciprofloxacin	0.03	0.06	0.008-0.5	2,208
Levofloxacin	0.03	0.06	0.008-0.5	2,028
Tetracycline	128	128	32->128	2,188
Doxycycline	32	32	8-128	536
Chloramphenicol	1	1	0.5-2	2,137
Azithromycin	0.03	0.25	0.008-1	2,185
Rifampicin	0.004	0.008	<0.002-0.03	2,050

Complete investigation identifying underlying contamination issues has ruled any MDR *Legionella* spp. from this investigation. With only around 0.5% of isolates showing any resistance. Of the 11 isolates of that showed some resiatnce (one or two antibiotics), ten were *lpeAB*+ azithromycin resistant strains, one had an elevated ciprofloxacin MIC (but only slightly, as it grew one dilution higher than the MIC_{90}), one had an additional elevation of rifampicin MIC and two isolates had very high ampicillin MIC (one of which had a normal azithromycin MIC). These are shown in Table 45.

Table 45 Showing the resistant *Legionella* isolates

ID	ST	Ciprofloxacin	Azithromycin	Rifampicin	Ampicillin
34-29	1		0.25		
41-24	1717		0.25		
44-74	1		0.25		
44-76	1		0.25		
44-80	1		0.25		
45-24	1				128
45-02	1		0.125		
53-25	1		1		
45-01	1		0.125		
24-36	1		1	1	
49-13	84	0.125	0.125		128

Following extensive examination of potential non-*Legionella* bacterial contamination, there were no remaining *Legionella* spp. that were resistant to gentamicin, chloramphenicol, or levofloxacin. (Isolate 49-13 with elevated ciprofloxacin MIC was within the MIC_{90} for levofloxacin).

6.11 Discussion

Postulating as to the reasons for the absence of AMR in *Legionella,* it is primarily due to the lack of person-to-person transmission preventing the acquisition and subsequent transmission of resistance. Hence resistant strains of *Legionella* are exceedingly rare. BCYE-α and LASARUS are nutritious and non-selective therefore there are ongoing risks of contamination with non-*Legionella* species. The addition of selective antibiotics such as vancomycin was not investigated due to the effects that sub-inhibitory dosing can have on bacterial growth which would have skewed MIC testing, as has been shown other studies (Descours et al. 2014; Scaturro et al. 2020). Growth on cysteine-free BCYE-α would have probably identified the MDR co-colonising contaminates earlier but was avoided to reduce cost and plastic wastage.

Additional investigations into whether LASARUS would have supported *Legionella* growth without additional cysteine would have been of benefit, as well as a more detailed investigation of the brown colouring seen in the *Legionella* plates as well as the representation of the "cut-glass" colony morphology as seen on BCYE-α agar and a more in-depth investigation as to their presence in other media.

7 General Discussion

There are 63 species within the genus *Legionella* as accepted by the German DSMZ (https://lpsn.dsmz.de/genus/Legionella , 09/12/2021) the majority of which have been isolated only from environmental, rather than from clinical, sources.

Legionnaire's disease is found in large outbreaks, sporadic, community-associated, travel-acquired or nosocomial infections (Fields et al. 2002). It is a significant cause of adult mortality caused primarily by *Legionella pneumophila*. Categorised as either travel, hospital or community acquired pneumonia, with a European case rate of around 2.2/100,000 people and 300 diagnosed from January to September 2020 in England and Wales (UKHSA 2020), in confirmed cases this disease has a mortality around 20% but up to 40% in nosocomial cases (Herwaldt et al, 2018). During pneumonia, *Legionella* spp. invades and replicates within alveolar macrophages, which limits effective therapy to those antibiotics with intracellular penetration (fluoroquinolones, macrolides rifampicin, tetracyclines and trimethoprim) and excludes those with poor penetration (aminoglycosides, penicillin's) (Bongers et al. 2019). This is especially important as treatment for LD is often empiric based of diagnosis for travel, community or hospital acquired pneumonia and as such antibiotics which will not function on an intracellular organism may inadvertently be used (Lim et al. 2015).

Growing *Legionella* in the laboratory requires the use of specialist media containing (amongst other ingredients) activated charcoal, a potent toxin chelator necessary for growth. When testing for antimicrobial resistance in *Legionella*, the incorporation of activated charcoal skews the results, as it is shown by Portal *et al* (Portal et al. 2021a).

When analysing the published literature variations in methodologies between agar dilution and E-strip on BCYE-α versus activated charcoal-free BMD (as described by (Vandewalle-Capo et al. 2017)), it was immediately apparent that the breadth of ranges was being skewed by the lack of methodological concordance (Portal et al. 2021b).
As such I postulated that resistance in *Legionella* was under-reported and obfuscated by the chelating effect of activated charcoal and merging of non-compatible datasets. If *Legionella* possessed unreported antimicrobial properties or harboured known resistance genes, then elucidating this was of critical importance, even if these genes were clinically irrelevant for *Legionella*, such as is the case for gentamicin or ampicillin, the *Legionella* could still act as a host and distributor of such resistance genes, contributing passively to the growing AMR epidemic. Antibiotic resistance is a global health crisis, with the potential to cause 50 million deaths and 100 trillion USD loss per annum by 2050 (O'Neill 2016). To date the largest investigation into resistance in *Legionella* was published by Garcia *et al* in 2000, which investigated 271 *Legionella* isolates, but only against three antibiotics, two fluoroquinolones (ciprofloxacin and levofloxacin) and a macrolide (erythromycin) (García et al. 2000).

Given the limitations of antibiotics available to treat legionellosis, it is particularly important to be able to identify emerging resistance to those therapeutics that remain as potential candidates if resistance does appear in the current drug regime or if patients are not responding to empirical treatment. International guidelines, ECOFF values, well-validated methodologies and control strains validated in multiple laboratories are all absent for antimicrobial susceptibility testing of *Legionella*. My thesis aimed to answer these AST question in a large scale study.

When I initially attempted to determine the AST profile of *Legionella* it became apparent that the current culture methods were unsuitable for testing at scale. This led to the creation, validation and patenting of a new media formulation LASARUS, for screening a large cohort of *Legionella* isolates.

7.1 Follow up and work not yet done

7.1.1 Legionella sainthelensi, Legionella longbeachae and putative species of Legionella originating from the Czech Republic

An investigation into a single case of *Legionella sainthelensi* and 15 cases of *Legionella longbeachae* from England and Wales were re-analysed, using a novel MinION protocol developed in UKHSA to detect bacterial DNA from a BAL sample. This was in essence successful and *Legionella sainthelensi* and *Legionella longbeachae* DNA was discovered through sequencing. The challenges associated with this method primarily revolved around the depletion of human DNA. This is a critical technical requirement: as 99% of the DNA present in an unprocessed sample is likely to be of human origin, leaving only the remaining 1% to cover analysis of all present respiratory bacterial species. Even if an invasive pathogen is outcompeting commensal bacteria, sequencing was not possible in sufficient depth to elucidate bacterial species. Therefore, depletion of human (methylated) DNA was the most important aspect of this sequencing. A saponin depletion method removed enough human DNA to allow sequencing of bacterial DNA. Due to UKHSA data protection, the presence of human DNA sequences (even when not being investigated or patient identifiable in anyway) required physical confinement to the UKHSA site. This meant all analysis were performed at the UKHSA facility in Colindale, London where work was carried out in conjunction with the lead scientist and bioinformatician on this project. This was a significant restriction even before the pandemic, but during and after the COVID-19 pandemic, all work on this project came to a halt as UKHSA policy required all staff members to work from home where at all possible. Once travel and in-person meetings are permitted, this project will be completed and written up

describing the clinical case, the initial diagnosis and treatment, alongside the novel sequencing based metagenomic diagnostic tool. An additional *Legionella* spp. Project that involved characterisation of 28 potential novel environmental *Legionella* species, archived and transferred from the Czech Republic to UKHSA. Analysis carried out at UKHSA included electron microscopy, qPCR of *mip* gene and other *Legionella* genes, investigation of fluorescence under UV light. In Cardiff I began a combined whole genome sequencing on these strains using the hybridised Illumina and MinION approach. I also investigated the MIC to the antibiotics mentioned in results chapter 3 as well as investigating their reactions to cations. This work was interrupted by COVID-19 and once in-person meeting and visiting researchers are allowed back into UKHSA Colindale I will be able to combine the analysed results for this work and publish.

7.2 What I would do differently

In retrospect, as soon as potentially resistant isolates were flagged by my screening methodology, I should have tested them for higher concentrations of antibiotics and immediately sequenced them. I also should have selected the highest MIC colony for sequencing, which would have eliminated spending time collecting and analysing what appeared to be multi-resistant *Legionella* spp. rather than co-cultured bacterial species. Due to the COVID-19 pandemic, I lacked access to the MALDI-ToF as prior to COVID-19 our access was dependant on NHS resources. This also reduced my ability to identify non-*Legionella* species contaminants as standard operating procedure prior to COVID-19 routinely required bacterial species confirmation using the Bruker BioTyper database for colonies with elevated MICs, without having to sequence them. Combined these improvements would have helped solve some of the main challenges I faced within this project. Adding a cysteine free plate in every MIC batch as a *Legionella* confirmation plate would also, in retrospect, have been of benefit and would have removed some but not all of the contaminant isolates within my dataset.

7.3 Future work

7.3.1 Chromogens

This thesis has shown the invention of a novel patented a media that was capable of growing *Legionella* species that was clear and free of charcoal. Future work identifying chromogenic biochemical metabolites for putative species identification (i.e. such as UTI chromogenic agar utilises) would give a significant advantage to LASARUS over BCYE. Beyond aiding AST investigation, chromogenic species identification could differentiate *Legionella* spp. in both environmental and clinical samples. This would change the way in which *Legionella* are investigated and diagnosed globally, reducing time to diagnosis and thereby could positively impact on patient care. This would be important too if chromogenic substrates could differentiate biochemically between clinically important *L. pneumophila, L. longbeachae, L. anisa* or *L. bozemanii,* compared with the other 59 species which are, except in very rare circumstances environmental.

Currently these samples are differentiated by having to use both a cysteine and cysteine free BCYE-α plate, which have significant disadvantages not least is the increased cost in materials and time. Reducing plate numbers by alleviating this as a requirement through chromogenic LASARUS would therefore be of financial importance too. Notwithstanding the environmental considerations of plastics use, something of ever-increasing importance in a field which all too often disregards its environmental impact. Collaborations with private corporations, as well as UKHSA have already been started and this work is currently ongoing.

7.3.2 Cation/Biofilm work

This research has shown that the standard practise of creating McFarland standards of *Legionella* species in 0.85% saline solution, which is in the current EUCAST guideline has a negative impact on *Legionella* growth. This project has shown that cations including NaCl are highly toxic to *Legionella* and a possible recommendation would be to prepare the *Legionella* suspension work in water devoid of additional cations used to preserve homeostasis in other species. These observations spurred speculation about the effect of 'hard' vs 'soft' water on *Legionella* prevalence with the hypothesis that water containing more cations would inhibit *Legionella* growth and therefore one would see reduced cases of *Legionella* produced at sites with hard water (naturally occurring increased calcium and magnesium ion presence). Personal communication with Gary Hogben and the team at Feedwater Ltd (an environmental water testing company), however, have found the opposite correlation: hard water areas have greater occurrence of positive *Legionella* spp. detection. They speculated that the presence of hard water increases the calcium depositions within water pipes, which in-turn allows greater capacity to support biofilm growth. Furthermore, the intracellular nature of *Legionella* (i.e. environmental expectation of requisite growth within amoeba species) also adds unknown variables. The effects of increased calcium and magnesium on an amoeba biofilm model to accurately map the environmental colonisations would be another topic of value for future work.

7.4 Limitations of LASARUS

The development of LASARUS, a charcoal-free solid medium for *Legionella,* that generated concordant MIC values when compared with those determined using the BMD, provides a reliable solid medium alternative to BMD for AST in *Legionella*, which is also more applicable in a high-throughput lab and avoids some of issues inherent with a BMD.

However, it is important to note that *in vitro* testing is only the first step in the eventual goal of determining clinical resistance thresholds and does not take into account *in vivo* drug pharmacokinetics and pharmacodynamics. *In vitro* antibiotic susceptibility testing also does not take into consideration cellular penetration; important as *Legionella pneumophila* is an intracellular pathogen. An example of the importance of *in viv*o testing is demonstrated by the efficacy of doxycycline, which despite having an elevated in vitro MIC_{50} and MIC_{90} of 32µg/mL was as effective as erythromycin against *L. pneumophila* in an animal model study (Edelstein et al. 1984). Moreover, tigecycline has also been shown to have a BMD MIC of 4µg/mL, yet was as effective as azithromycin for preventing death in a LD guinea pig animal model study (Edelstein et al. 2003).

7.5 Recommendations

7.5.1 Standardisation of inoculation time and dilution in water.

This work has demonstrated the need for consistent standardisation, not just in the media used, but also the methodology. Normalising the amount of time isolates are cultured for, in non-fastidious organisms, such as *E. coli,* is easy and defined almost universally as overnight. With slow growing organisms, the effect of a small change in method can potentially have large impacts on the results of AST. This is most true with bacteriostatic compounds where after several days a bacteria may grow through the inhibitory activity of the antimicrobial. In this work all isolates were grown for five days, as this ensured that an MIC could be read for isolates that grew more slowly. As well as standardising the amount of time isolates are grown for, normalising the inoculation concentration (a dilution of 0.5 McFarland standard suspended in molecular grade water) needs to be standardised for future studies.

7.5.2 Standardisation of methods and internationally agreed recommendations

The MIC dataset of existing publications presented in (Portal et al. 2021b) and in the attached appendix shows a wide variation in values and methods utilised for susceptibility testing. Currently, gradient MIC strip testing on BCYE-α agar is the methodology recommended by EUCAST (EUCAST 2016). The inefficiencies of this methodology have been highlighted repeatedly within this work but reiterating the sequestration of antibiotics by the activated charcoal, as well as variations in charcoal content from plate-to-plate (due to suspension settling during pouring), lead to these problems, as has been previously suggested by: (Ruckdeschel and Dalhoff 1999; Bruin

et al. 2012). Publications arising from work done in this thesis (Portal et al. 2021a) showed that gradient strip testing and BCYE-α agar dilution methodologies gave higher MIC values than the BMD used in Vandewalle-Capo et al. 2017.

To address these challenges in conjuncture with the international *Legionella* community an international position paper was published, including representation from many international *Legionella* reference laboratories, as well as from the EUCAST Steering Committee and the CDC (Portal et al. 2021b). This agreed upon the following recommendations, due to the lack of comparable data and the varied approaches and methodologies in use across the globe to address this topic;

1. Gradient strip testing on BCYE-α agar should be discontinued as the recommended EUCAST methodology, due to higher (and more variable) MIC results when compared with BMD.
2. BCYE-α agar should no longer be used for serial antibiotic dilution MIC determination for *Legionella* due to higher MIC results and antibiotic sequestration.
3. Future studies to develop and standardise BMD as the gold standard for determination of susceptibility of *Legionella*, to enable interpretation and standardization of more accessible concordant methodologies, such as charcoal-free media (e.g., LASARUS).

7.6 Advancement of the field beyond this research

International treatment recommendations for patients with *Legionella* infection are also inconsistent, often providing differing guidelines and regimens (Lim et al. 2015; NICE 2019b; NICE 2019a) and employing variable defined breakpoints for assigning susceptibility/resistance phenotypes. Historically, antibiotic resistance in *Legionella* has not been a concern. However, reports of the *lpeAB* genes encoding a macrolide efflux pump (Vandewalle-Capo et al. 2017) and single point somatic mutations in *L. pneumophila* 23S rRNA (Shadoud et al. 2015) have increased, which mediate intermediate to high levels of antimicrobial resistance (azithromycin and ciprofloxacin respectively). Moreover, a documented tetracycline resistance gene in *Legionella longbeachae tet(56)* (Forsberg et al. 2015) (the most common source of legionellosis in Australia and New Zealand), highlights the need for standardization and validation at an international level. However, enabling evidence-based treatment guidance both nationally and internationally is required and for patients with persistent infection. That understanding the MIC may be of clinical benefit. This is confounded by the unknown significance of increases in MIC in clinical practice and how this relates to epidemiological cut-off values, which have not been assigned or agreed for *Legionella* species to date.

Part of this project's aim was to develop internationally agreed ECOFF values, which could populate the European database.

This would ensure that global access to a 'normal' range, making elucidation of resistant isolate significantly easier. To achieve this however it is vital that work is repeated in not just one but multiple laboratories and is in fact a requirement for

ECOFF before they will agree to a defined ECOFF value. (https://www.eucast.org/mic_distributions_and_ecoffs/). As such the next stage of this work is to set up an international collaboration, consisting of several laboratories with the capacity to screen *Legionella* at scale, to develop a consensus standard operating procedure, define ECOFF values and develop consensus on antibiotic testing of strains of clinical relevance. This will encourage antimicrobial susceptibility testing for *Legionella* in global surveillance enabling the detection of resistance.
When these phenotypically resistant *Legionella* isolates are identified they should be comprehensively analysed using WGS and other complementary methods in order to identify new and emerging mechanisms underlying resistance in the Legionellaceae.

Currently, performing *in vitro* antimicrobial susceptibility testing is unlikely to be useful in clinical practice for the majority of patients, due to the time taken for a culture to MIC determination and that not all infected patients have recoverable *Legionella* isolates, apart from in rare cases of a potentially recurring *Legionella* reinfection (Cassell et al. 2021), a problem which would be exacerbated by AMR. There is the potential for screening to be of use in the search and modification of treatment in relation to *lpeAB* positive patients. However, with the potential to select for *Legionella* and with the addition of chromogens only available with a novel clear media (LASARUS) the initial screening has the potential for automation, and using colour recognition in robotics, means analysis can be performed on a large scale with a reduced need for highly skilled human intervention. This would be of significant advantage in day-to-day screening for water testing companies and, may also prove vital in controlling large outbreaks of *Legionella*.

This work has shown the first mass screening of *Legionella* to a panel of antibiotics mapped to the internationally agreed gold standard. This thesis has not only developed, validated and patented a novel media followed by AST screening of over 2,100 isolates but has also produced the first international viewpoint on antibiotic resistance in *Legionella*.

Which I hope will improve international consensus and quality of microbiological antibiotic susceptibility testing, positively impacting understanding of the level and mechanisms of resistance to antibiotics in *Legionella.* Ultimately providing more accurate data which can be used internationally to inform clinicians treating those with Legionnaires' disease.

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