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Broth microdilution protocol for determining antimicrobial susceptibility of *Legionella pneumophila* to clinically relevant antimicrobials

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ABSTRACT

Currently there is no detailed, internationally agreed protocol defined to evaluate antimicrobial susceptibility testing (AST) for *Legionella pneumophila* (required to establish epidemiological cut-off value or "ECOFF" boundaries); therefore, antimicrobial resistance in these isolates cannot be defined. AST methods utilising media containing activated charcoal as an ingredient, to enable *Legionella* growth, are unreliable as noted in an internationally authored opinion paper and a new gold standard is required. Here we define a detailed protocol for broth microdilution (BMD) using defined cell culture collection-deposited control reference strains (Philadelphia-1 and Knoxville-1) as well as two accessible reference strains with moderately (*lpeAB*-carrying) and markedly (23S *rRNA* mutation-carrying) elevated azithromycin minimum inhibitory concentration (MIC). The

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defined protocol enables up to eight *L. pneumophila* strains to be set up on a single 96-well plate per antimicrobial tested. Initial ranges to routinely capture an MIC for these reference strains using clinically relevant antimicrobials azithromycin (0.01–0.25 mg/L), levofloxacin (0.008–0.03 mg/L), lefamulin (0.01–2 mg/L), rifampicin (0.0002–0.0008 mg/L) and doxycycline (0.25–16 mg/L) following incubation for 48 h at 37 °C in a shaking incubator have been empirically determined. Establishment of this internationally agreed protocol sets the scene for the next step: validation and comparison of antimicrobial ranges between international *Legionella* reference laboratories to establish putative resistance cut-off thresholds for these clinically relevant antimicrobials.

1. Introduction

Legionella spp. are a Gram-negative bacterial parasite of amoebae that are commonly found in manmade water systems (Fields et al., 2002; Zhu and Liu, 2023). *Legionella* can cause human diseases ranging from a mild flu-like upper respiratory tract illness called Pontiac fever through to a, sometimes fatal, atypical pneumonia called Legionnaires' disease (Fields et al., 2002). *Legionella* was first identified in 1977 (Fraser et al., 1977) and to date there are 69 named separate species of *Legionella* in the NCBI taxonomy browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi)); however critical analysis of the available data 63 *Legionella* species (and three subspecies) meet the minimum valid definition criteria according to the rules of the International Code of Nomenclature of Prokaryotes (personal communication from UK Health Security Agency). At least 25 of these species have been isolated and/or associated directly with human disease (Sharaby et al., 2019). *Legionella pneumophila* is the most clinically relevant species responsible for 90 % of Legionnaires' disease (Gattuso et al., 2022; Massip et al., 2017), which has a mortality rate ranging from 4 to 40 % (Rello et al., 2024), the highest usually associated with infections in immunocompromised patients (Vandewalle-Capo et al., 2017; Viasus et al., 2022). To date, there are 16 established *L. pneumophila* serogroups (SG; with SG1 having the highest disease association association and is implicated in most reported outbreaks) (Fields et al., 2002). Legionellosis occurs due to inhalation of *Legionella*-containing aerosols (Fields et al., 2002). Once inhaled, *Legionella* is phagocytosed by alveolar macrophages (Zhu and Liu, 2023). Similarities between the normal amoebic host cell and macrophages allow *Legionella* to inhibit phagosome-lysosome fusion and thrive within macrophages (Newton et al., 2010; Strassmann and Shu, 2017). This intracellular environment presents a hurdle for many antimicrobials so effective treatments require intracellular penetration as noted with antimicrobials such as macrolides, tetracyclines, lefamulin, fluoroquinolones and rifampicin (Sharaby et al., 2019). Macrolides or fluoroquinolones are recommended as primary antimicrobial treatments by the Infectious Disease Society of America (Jasper et al., 2021) and the most recent update for European guidelines (Viasus et al., 2022) due to their efficacy against intracellular L. *pneumophila in vitro*. For immunosuppressed patients, fluoroquinolone antimicrobials are advised to treat Legionnaires' disease due to the potential interaction of macrolides with immunosuppressive agents (Fields et al., 2002; Ruckdeschel and Dalhoff, 1999). However, even with early intervention using these antimicrobials, approach with empirical treatment is still not completely effective as evidenced by the high mortality rate (Fields et al., 2002).

Acquired antimicrobial resistance remains a critical global health issue. Multiple *in vitro* studies have shown *L. pneumophila* can readily acquire greatly elevated MICs (<512 mg/L) to macrolides, fluoroquinolones and rifampicin following repeated sub-inhibitory challenge with these antimicrobials (Almahmoud et al., 2009; Nielsen et al., 2000). Recently, an environmental strain of L. *pneumophila* SG1 isolate with a high macrolide MIC was identified (erythromycin and azithromycin MICs $\geq$ 1024 mg/L) mediated by a A2052G mutation in three copies of the 23 s *rRNA* operons (Ginevra et al., 2022). Consistent elevation in azithromycin MICs has also been noted in isolates carrying the *lpp2879* (*lpeA*) –*lpp2880* (*lpeB*) operon, that encode constituents of a tripartite efflux pump akin to the AcrAB-TolC pump found in *Escherichia coli* (Massip et al., 2017; Natås et al., 2019; Vandewalle-Capo et al., 2017). *lpeAB* reduces macrolide susceptibility presumably by actively expelling intracellular macrolides before they can inhibit translation at the ribosome (Massip et al., 2017). Furthermore, mutations upstream of the *lpeAB* genes have been found to further reduce susceptibility of clinical isolates to macrolides (Natås et al., 2019). Elevated fluoroquinolone MIC has also been observed in clinical isolates due to a point mutation at position 83 of *gyrA* in a patient being treated with ciprofloxacin (Bruin et al., 2014; Jonas et al., 2003). A tetracycline deconstructase flavoenzyme has also been identified in environmental *Legionella longbeachae* encoding the *tet*(56) gene, which has been speculated to degrade members of the tetracycline family (Forsberg et al., 2015). The identification, characterisation and surveillance of potential resistance mechanisms within *Legionella* is essential as delay in effective therapy is associated with significant increases morbidity and mortality (Heath et al., 1996) but is rarely performed in clinical settings.

The lack of international Clinical and Laboratory Standards Institute (CLSI)/ European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines for *L. pneumophila* antimicrobial susceptibility testing (AST) methodologies complicates interpreting AST datum and makes it implausible to accurately identify "resistance" for this species. In 2021, the majority of European *Legionella* reference laboratories and the Centers for Disease Control USA called for standardisation of *Legionella* AST using widely-available reference strains (Portal et al., 2021a). Standardisation of an AST methodology is the first step towards defining EUCAST epidemiological cut-off (ECOFF) values to distinguish susceptible wild type isolates from those with acquired resistance. Only then will resistance prevalence for environmental and clinical *L. pneumophila* be possible to survey; hopefully informing therapeutic guidelines and aiding development of rapid resistance assays (Kahlmeter and Turnidge, 2022). AST for assessing the susceptibility profiles of *L. pneumophila* has been approached using several different *in vitro* methods including agar diffusion, agar dilution, antimicrobial gradient strips and broth microdilution (BMD) (Portal et al., 2021b). With the exception of LASARUS agar and BMD, most AST methods produce conflicting and falsely elevated MICs due to the incorporation of activated charcoal in the solid. Activated charcoal was deemed essential for *Legionella* growth, as it chelates growth-inhibitory compounds that are produced from autoclaving agar but subsequently elevate the MIC by up to 5 times, relative to BMD, as the charcoal also absorbs antimicrobial compounds in the agar to varying degrees (Portal et al., 2021b).

Here, we report a BMD method for *Legionella* AST as the possible future gold standard, following evaluation with two susceptible culture-collection deposited *L. pneumophila* reference strains (Philadelphia-1 and Knoxville-1) and two non-susceptible well-characterised strains with elevated azithromycin MIC (the 23S *rRNA* mutant (Ginevra et al., 2022), and an *lpeAB*-carrying strain). We also define a protocol to generate testing for five clinically relevant antimicrobials (azithromycin, doxycycline, lefamulin, levofloxacin and rifampicin) with putative concentrations ranges that will capture susceptible isolates to clearly identify isolates with abnormally elevated MICs within a single 96-well plate. This method can be expected to generate consistent results for future international validation.

2. Methods

Fig. 1 shows a schematic of how the BMD susceptibility testing

protocol was set up.

2.1. L. pneumophila strains and preparative BCYE-α culture

Four *L. pneumophila* SG1 strains were analysed including two susceptible culture-collection deposited *L. pneumophila* reference strains (Philadelphia-1 National Collection of Type Cultures (NCTC) catalogue number: 11192; Knoxville-1 NCTC: 11286) and two relevant, characterised strains with elevated azithromycin MICs (the 23S *rRNA* mutant Moulins strain NCTC: 15116; and an *lpeAB*-carrying strain PHELPN45–24; NCTC: 15115). All strains had been archived at −80 °C in glycerol cryobead vials and used one passage after recovering from frozen stocks. The first two isolates arose from the initial characterisation of *L. pneumophila* (Brenner et al., 1979) and there is no accurate way to determine their cummulative passage number, while the latter two more recent isolates were used between passage number 4–10).

Buffered charcoal yeast extract (BCYE-α) plates were prepared by autoclaving 450 mL H_2O with 12.5 g of *Legionella* agar base (Sigma Aldrich 74,303) and cooled in a water bath set to 55 °C before adding BYE-α supplement (VWR 84726.0001, contains L-cysteine) and 2 mL of amphotericin B (Sigma Aldrich A2942; a 250 μg/mL solution in deionised water kept at −20 °C until required). 20 mL of BCYE-α solution was poured per 90 mm plate. Negative control BCYE-α plates were also made using L-cysteine-free BYE-α supplement (VWR 84727.0001). Viable inoculation cultures of *L. pneumophila* were generated by subculturing on BCYE-α agar plates containing L-cysteine incubated for 72 h at 37 °C in a humidified chamber. Failure to grow on L-cysteine-free BCYE-α agar plates inoculated in parallel was used to identify contamination.

2.2. Buffered yeast extract (BYE-α) broth

One vial of BCYE α-growth supplement (VWR 84726.0001; containing L-cysteine) was resuspended in 50 mL of sterile H_2O and added to 5 g of vitamin-enriched yeast extract (Sigma Aldrich 07533) and 2 mL amphotericin B (Sigma Aldrich A2942). This solution was made up to 500 mL using sterile, deionised H_2O and pH adjusted to pH 6.95 ± 0.02 using 50 mg/mL KOH before being filter sterilised with a 0.22-μm vacuum filter (Corning 430,769) and stored at 4 °C. The presence of amphotericin B is optional and is primarily included to maximise the shelf life of BYE-α, stored at 4 °C for up to a month, if necessary.

2.3. Antimicrobial preparation

Five clinically-relevant antimicrobials were evaluated: azithromycin, doxycycline hydrochloride, lefamulin, levofloxacin hydrochloride, and rifampicin as detailed in Table 1. Antimicrobial stocks

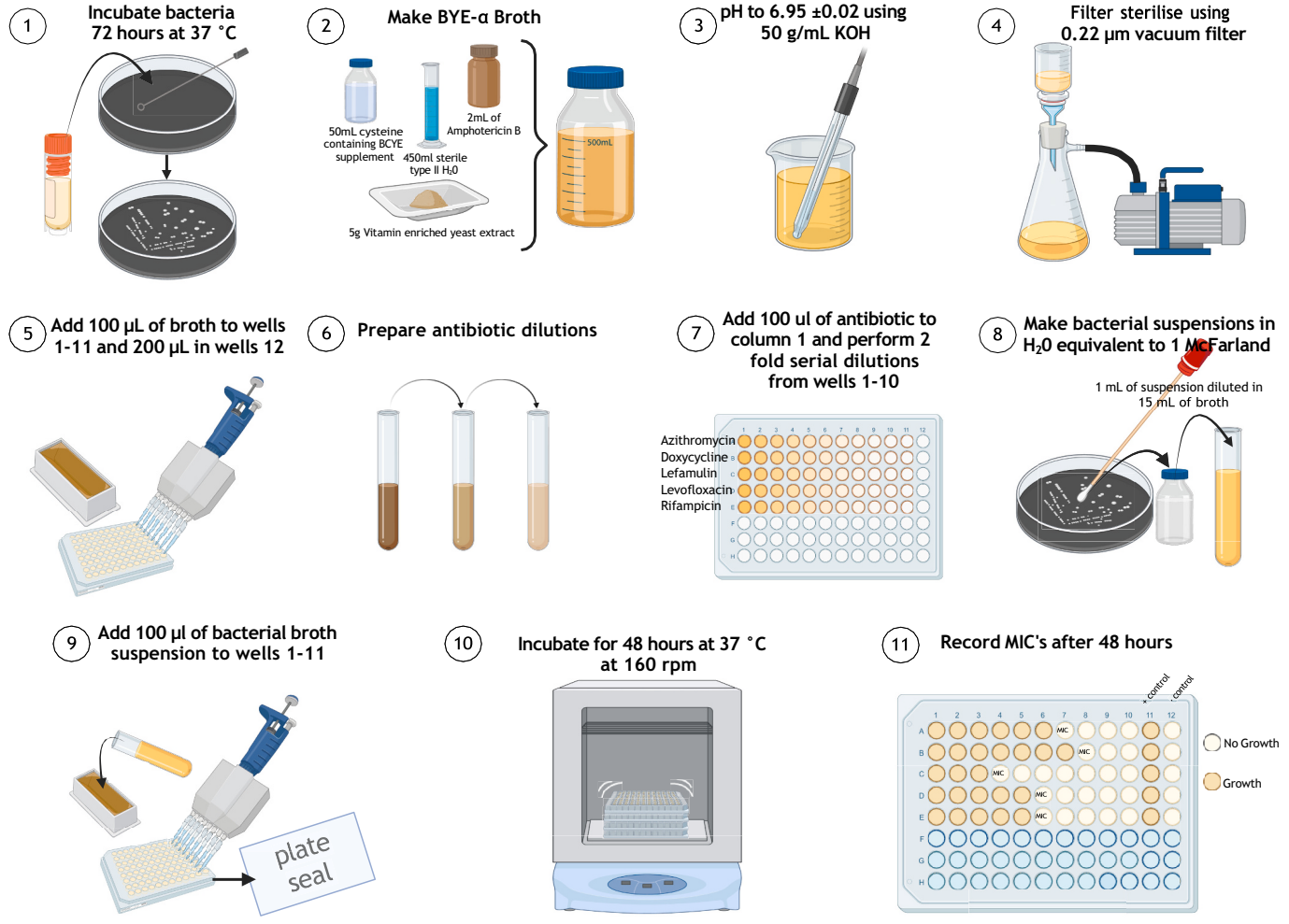

Fig. 1. Schematic representation of the AST protocol. Bacteria were preparted on BCYE-α at 37 °C for 72 h. After preparing broth the pH was adjusted to 6.95 ± 0.02, filter sterilised and used to prepare 96-well plates containing antimicrobial dilutions. Antimicrobials were serially diluted in the 96-well plate from wells 1–10. A one McFarland standard was prepared and diluted in 15 mL broth for each *L. pneumophila* strain, 100 μL of which was added to wells 1–11. Plate seals were applied before incubating at 160 rpm 37 °C (±2 °C), in a non-humidified incubator, for 48 h before recording MIC results. Accompanying video at https://youtu.be/ALva0i9HBsY Figure generated with BioRender agreement number DL26ZG0D5I.

Table 1
Antimicrobial supplier, purity, stock and final testing concentrations.

Antimicrobial	Supplier	Purity	Antimicrobial Stock concentrations (mg/L)	Concentration range in 96-well plate (mg/L)
Azithromycin	Apollo Scientific CAS No.: 83905–01-5	98 %	16	4–0.008
Doxycycline hydrochloride	Apollo Scientific CAS No.: 10592–13-9	98 %	256	64–0.125
Lefamulin acetate	Nabriva Therapeutics CAS No.:135063–82-6	88 %	16	4–0.008
Levofloxacin hydrochloride	ChemCruz CAS No.: 177325–13-2	90 %	0.5	0.125–0.00025
Rifampicin	Apollo Scientific CAS No.: 13292–46-1	98 %	0.5	0.125–0.00025

Legend: Purity as defined by the supplier for the batch utilised in these experiments.

were prepared daily at a concentration four-fold higher than the highest concentration in the testing range, adjusting for purity as provided by the manufacturer. Rifampicin was initially dissolved in 500 μL Dimethyl sulfoxide (DMSO) before dilution in 4.5 mL of sterile, deionised H_2O. Azithromycin was added to 4 mL of sterile, deionised H_2O before the addition of 0.1 mol HCL to solubilise, then made up to a final volume of 5 mL. Detailed antimicrobial stock preparation methodology is provided (Supplementary Fig. 1).

2.4. BYE-α broth microdilution method for MIC determination

Each well of a sterile flat-bottomed 96-well plate received 100 μL of BYE-α broth, except for negative control wells in column 12, which received 200 μL of BYE-α broth. The first column received 100 μL of each antimicrobial stock (at four-fold the required final concentration), and a twofold serial dilution was performed by transferring 100 μL to the adjacent column, stopping at column 10, where 100 μL was discarded. The four control strains were routinely plated out on BCYE-α agar from frozen stock and incubated at 37 °C for 72 h before each repeat. Eight to ten single colonies were collected on a sterile cotton swab for each reference strain of *L. pneumophila* from the BCYE-α preparation plates and added to 3 mL purified H_2O (Oxoid) glass vials. Density was adjusted with addition of more single colonies or dilution with sterile water until the equivalent turbidity of one Mcfarland density was achieved (representing ~3×10^8 colony forming units (CFU)/mL). One millilitre of each bacterial suspension was then added to sterile tubes containing 15 mL of BYE-α broth solution (one for each reference strain), mixed, and 100 μL of bacterial broth suspension was added to columns 1–11 of the prepared 96-well plate containing the diluted antimicrobials (separate 96-well plate for each reference strain). Addition of bacterial suspensions to the 96-well plate should occur in less than 2 h of starting the one McFarland suspensions and transfer of sealed plates to the shaking incubator should occur in less than 30 min of addition of bacteria to the 96-well plates. This bacterial load was chosen as it was found to consistently grow in all wells in the absence of antimicrobials and represents 3-4 $\times 10^6$ CFU/mL per well when quantified by colony counting of dilutions on BYCE agar. Transparent microplate seals (Azenta 140,813) were applied to stop evaporation before incubating at 160 rpm (Luckham R100 rotatest shaker) at 37 °C (±2 °C). As plates are sealed to the atmosphere, no addition of CO_2 or humidification is required. Plates were observed at 24 h, without removing the plate seals, as any contaminants were expected to grow faster than *L. pneumophila*. MIC values were determined as the lowest concentration of antimicrobial at which no turbidity was observed after 48 h of incubation. To ensure turbidity in the highest antimicrobial concentration at 48 h was not due to contamination, this well was subsequently streaked onto an L-cysteine free BCYE-α plate and incubated for 72 h – failure to grow on this medium confirmed the turbidity was due to *L. pneumophila*.

2.5. Statistical analysis

Relevant statistical analysis was preformed using GraphPad Prism version 9.0.0. All graphs utilise Log_2 scale and geometric means with geometric standard deviations are shown. As data was not normally distributed, non-parametric analysis was performed using Kruskal-Wallis analysis with Dunn's post-hoc correction for multiple comparisons. Log_2 transformation of datum was not able to adjust data to be normally distributed as assessed by Kolmogorov-Smirnov test, therefore one-way ANOVA was not suitable. Statistical significance was presumed below $p \leq 0.05$ and descriptive statistics (geometric means, geometric standard deviations, 95 % confidence intervals, etc) were also calculated.

2.6. Ethics of reseach statement

No ethical approvals were required as all reference strains were obtained from NCTC. The authors also declare that they have no direct competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

3. Results

3.1. Legionella inoculum determination

To ensure that MIC determination would not be subject to false results caused by inconsistent growth of *L. pneumophila* at the bacterial load used under the culture conditions, the optimal bacterial inoculum that consistently established clear turbidity at 48 h with shaking incubation was determined. All four *L. pneumophila* reference strains were plated in a two-fold dilution of BYE-α broth from a one McFarland stock and the lowest concentration to establish turbidity at 48 h recorded (12 replicates), in the absence of antimicrobial compounds (Fig. 2). The Philadelphia-1 (Phil) strain was found on average to generate turbidity at the highest dilution (as low as 3000 CFU/well), when compared to repeats for Knoxville-1 (Knox) only generated turbidity at 3×10^5 CFU/well ($p < 0.01$) as well as the *lpeAB*-carrying strain ($p < 0.05$) While all L. *pneumophila* strains exhibited similar average inoculum requirements (within 10-fold of each other), there was a large intra-experiment range. To ensure consistent growth every time, a final inoculum of 3×10^6 CFU/well, approximately 10 times the highest concentration required for Knoxville-1, was chosen for subsequent experiments.

3.2. Shaking incubator requirement

Establishing reliable MICs requires clear and reliable demarkation between wells containing growth (turbidity) and no growth. Previous BMD methods have relied on point-bottom 96-well plates without agitation (Vandewalle-Capo et al., 2017); however we found that using flat-bottom 96-well plates combined with agitation at 160 rpm to reliably give turbidity representing growth and to be easier than other methods. Direct comparison found that incubation without agitation also delayed clear demarkation between growth and inhibition by 24 h for all four *L. pneumophila* strains (Philadelphia-1, Knoxville-1, *lpe*AB and the 23S mutant) for our reference antimicrobials (azithromycin, doxycycline, lefamulin, levofloxacin and rifampicin). Microbial turbidity and MIC values were easily assessed after only 48 h incubation

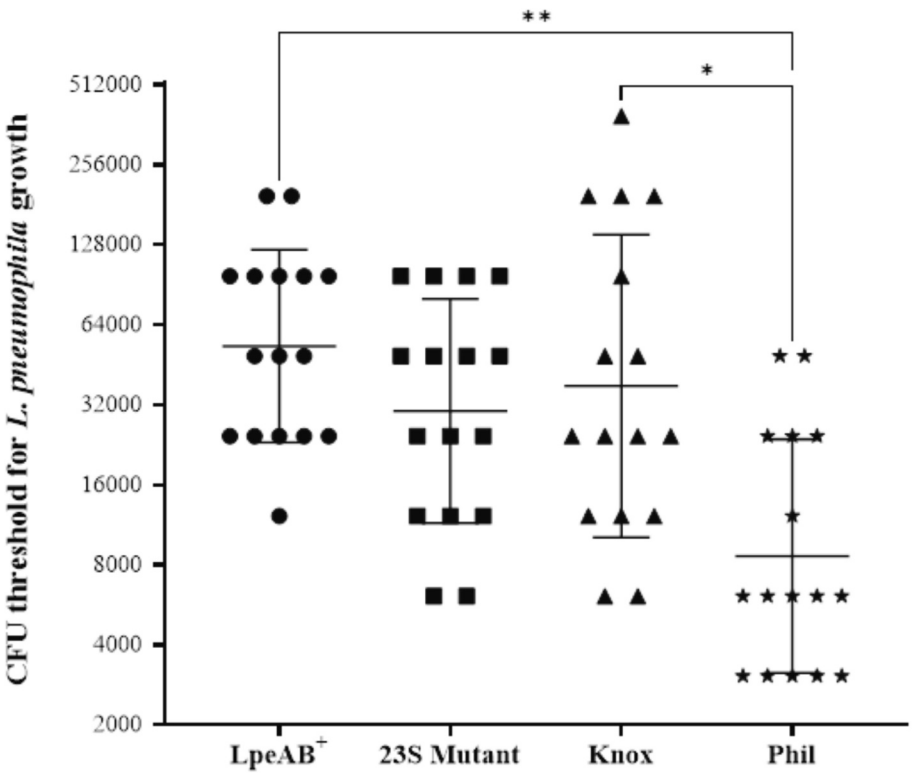

Fig. 2. Minimum CFU required for *L. pneumophila* growth after 48 h of shaking incubation. *L. pneumophila* strains Philadelphia-1 (star), Knoxville-1 (triangle), LpeAB^{+} (circle) and the 23S *rRNA* mutant (square) were serially diluted in BYEα broth in 96-well plates with concentrations ranging from 3×10^{6}-3×10^{3} CFU/mL. (Abbreviations; CFU colony forming units; Phil, Philadelphia-1 (NCTC: 11192); Knox, Knoxville-1 (NCTC: 11286); $p \leq 0.05$, *; $p \leq 0.01$, **. (Non-parametric Kruskal-Wallis analysis with post-hoc correction for multiple comparisons was utilised). Geometric mean and standard deviation are shown for combined data for three biological repeats each performed in quadruplicate.

in a shaking incubator (Supplementary Fig. 2).

3.3. Effects of incubation time on MIC results

The MIC values for azithromycin, lefamulin, levofloxacin and rifampicin remained relatively unchanged (increasing a maximum of one dilution) between 48 h and 72 h for all bacterial strains (Fig. 3). However, the MIC values for doxycycline consistently increased with length of incubation (differences between 48 and 72 h shown in Fig. 3), emphasising the requirement for achieving reliable MIC determination with the shortest (and standardised) incubation time for all bacterial strains.

3.4. Establishing susceptibility ranges for Legionella pneumophila strains

Twenty-three biological repeats of the AST method were performed on the four selected reference strains to establish consistency and strain-to-strain variation for clinically relevant antimicrobials. Philadelphia-1 and Knoxville-1 were selected as established, readily available susceptible reference strains. These strains showed very similar distributions for all repeats for all five antimicrobials, with no statistical significance in distributions (Fig. 4). However, the only available reference strains with known potential mechanisms of resistance (*i.e.* MIC elevation above normal distribution) were those for azithromycin: an *lpeAB*-carrying strain (PHELPN45–24; with moderate MIC elevation) and the 23S *rRNA* mutant (Moulins; markedly elevated)(Ginevra et al., 2022). The azithromycin MIC distribution for the *lpeAB*-carrying strain was significantly higher than for both control reference strains ($p \leq 0.0001$), although a minor overlap between the fringes of the distribution would make a clearly defined threshold difficult to establish (Fig. 4A). On the other hand, the isolate carrying the 23S *rRNA* mutation was consistently above the maximum 4 mg/L set for the test range, consistent with its MIC of 1024–2048 mg/L (data not shown). MICs for levofloxacin (Fig. 4D) and rifampicin (Fig. E) showed very little range between all four reference strains, indicating that mutations in either *gyrA* or *rpoB*, respectively, should be easily identified. Slight inter-repeat variability was observed for lefamulin, and some strain-to-strain differences were observed (Fig. 4C): Philadelphia-1 and the *lpeAB*-carrying strain showed equivalent susceptibility, while Knoxville-1 appeared consistently more susceptible in comparison. The lefamulin MIC for the 23S *rRNA* mutant strain was consistently elevated relative to all the other strains, but mutations in this region have been reported to give small but significant MIC elevation for lefamulin in other species (Spiller-Boulter et al., 2021). The MICs for doxycycline were found to be the most variable (Fig. 4B); however, this is likely directly related to the consistent increase observed with length of incubation noted in Fig. 3 above. The range of MICs for Philadelphia-1 alone ranged between 0.5 and 16 mg/L despite being performed on the same plates and recorded by the same experienced microbiologist as all the other antimicrobials to minimise variation in the 23 biological repeats.

4. Discussion

Here we report the design and testing of a BMD-based method for reliable and repeatable determination of *L. pneumophila* MICs. It utilises four reference strains (2 susceptible, 2 with increased macrolide MICs), all available from international cell collections or from the corresponding author, that we propose should be used as internal quality control standards to validate investigation of clinical isolates in the future. Optimisation of the method established a minimum bacterial load for consistent growth and determined that incubation of sealed plates in a shaking incubator gave a greater differential in turbidity

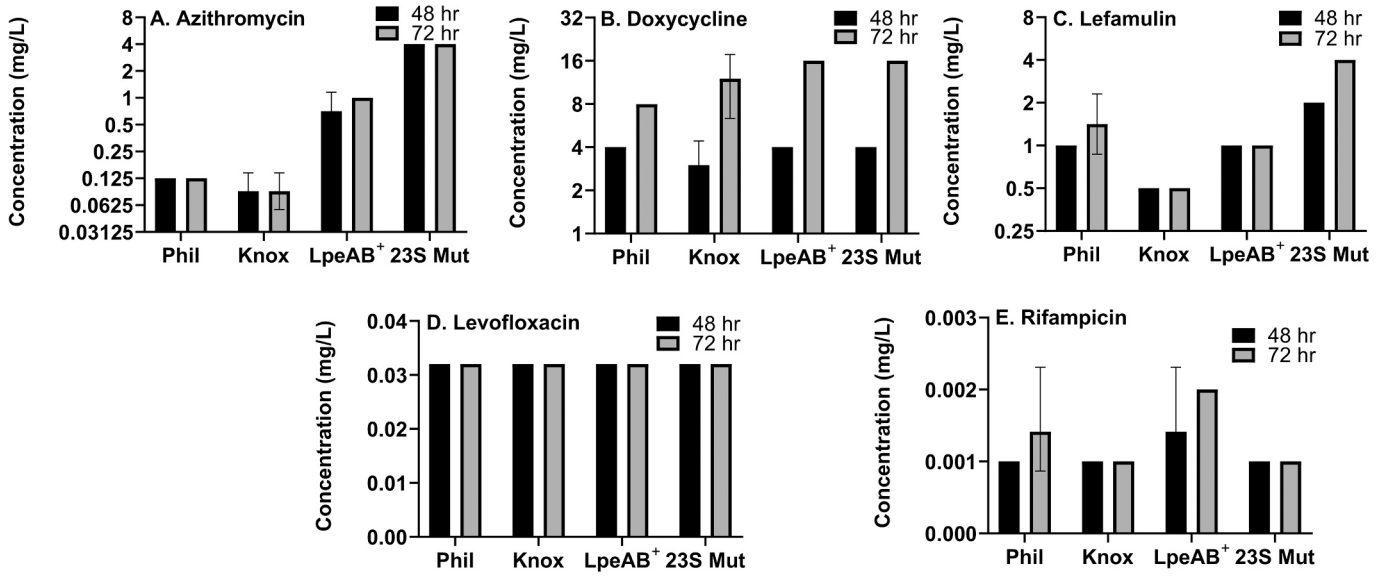

Fig. 3. Antimicrobial MICs at 48 and 72 h across four *L. pneumophila* strains using BMD.
MIC determination of defined cell culture collection-deposited control reference strains; Philadelphia-1 (Phil) and Knoxville-1 (Knox) and accessible elevated MIC reference strains; *lpeAB*-carrying (LpeAB^{+}) and 23S *rRNA* mutation-carrying strains against clinically relevant antimicrobials: azithromycin (A), doxycycline (B), lefamulin (C), levofloxacin (D) and rifampicin (E) using our standardised BMD method. MIC at 48 h (black) and MIC at 72 h (grey). Data represents the geometric mean ± geometric standard deviation for 3 replicates.

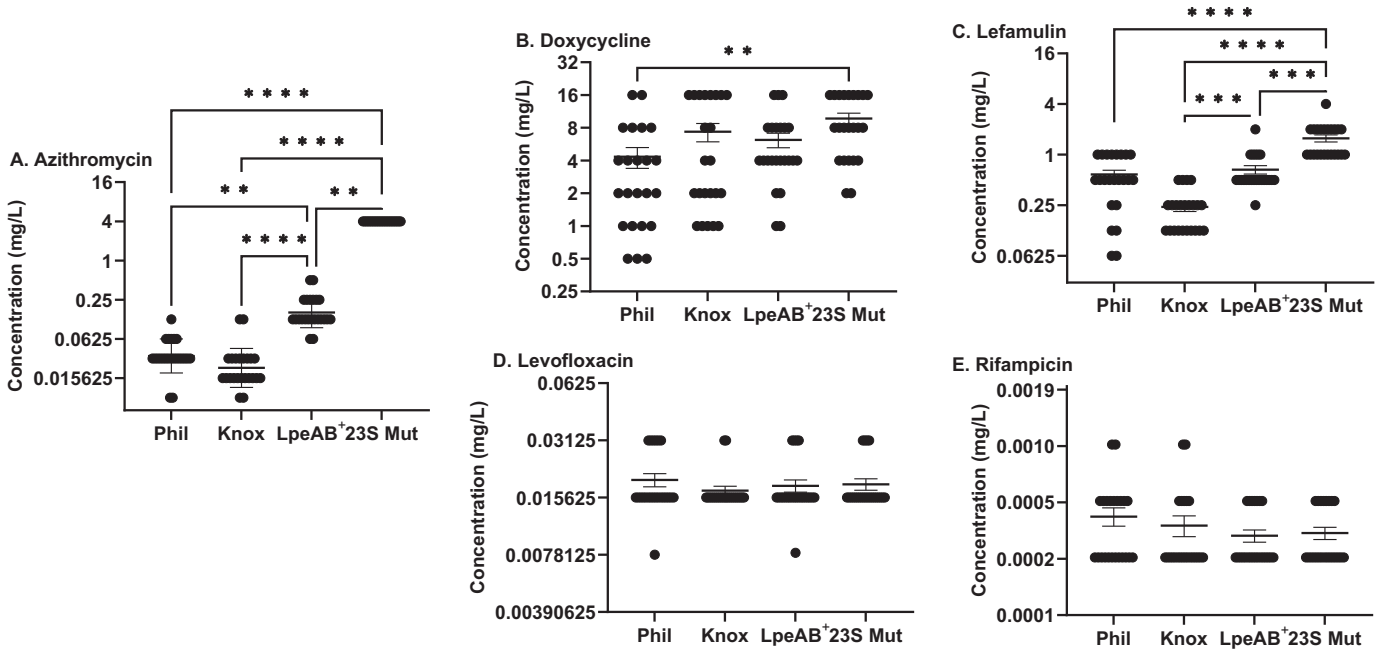

Fig. 4. Antimicrobial susceptibilities at 48 h compared across four *L. pneumophila* strains using BMD. MIC determination of control reference strains (Philadelphia-1 and Knoxville-1), *lpeAB*-carrying strain (PHELPN45–24; LpeAB^{+}, NCTC 15115) and an isolate with known 23S *rRNA* mutation (Moulins; Ginevra et al., 2022; NCTC 15116) for clinically relevant antimicrobials (azithromycin (A), doxycycline (B), lefamulin (C), levofloxacin (D) and rifampicin (E)) using the BMD method. Bars represent the mean with bars indicating the ±95 % confidence intervals for 23 replicates. (Abbreviations: Phil, Philadelphia-1 (NCTC: 11192); Knox, Knoxville-1 (NCTC: 11286); mutant, 23S *rRNA* mutant; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.01$, ***; $p \leq 0.0001$, ****. (one-way ANOVA with post-hoc Tukey test, corrected for multiple comparisons).

observation between growth and antimicrobial inhibition. Buffered yeast extract broth with cysteine and other supplements (BYE-α) is a well-defined pre-existing medium containing optimal nutrients for *L. pneumophila* growth and BMD is the preferred gold standard method by EUCAST and CLSI for MIC determination. Conditions that omit the presence of activated charcoal are essential, as the chelating effects of charcoal within growth media elevate the MIC to varying degrees for different antimicrobials (Bruin et al., 2012; Nielsen et al., 2000). Recently the international community highlighted the need for a standardised, reliable method that can be used to assess abnormal elevation of MIC values that could potentially impact therapy, including reference strains (Portal et al., 2021b).

Our method utilised flat-bottom 96-well plates and shaking incubation at 160 rpm which increased turbidity levels in wells with growth

and consequently reduced subjectivity in manual MIC determination. We only compared static incubation to circular-motion shaking at 160 rpm; therefore, this variable has not been fully interrogated for effect on turbidity. However, as long as the agitation does not result in contact between the plate seal and the broth, we do not anticipate the speed to be of importance, but it is unlikely that plate rocking would give a similar increase in turbidity. Previous BYE-α BMD methods have used point-bottom 96-well plates to take advantage of bacterial pellet settling to separate growth from inhibition (Vandewalle-Capo et al., 2017). However, we still found it difficult to differentiate between tiny bacterial pellets indicative of growth and occasional remnant representing initial inoculum. Shaking incubation always gave clear turbidity for wells with growth that will minimise subjectivity at 48 h.

Doxycycline MICs were unusually variable, despite the experimental design minimising confounding errors. Doxycycline MICs continuously climbed between 48 and 72 h (Fig. 4) and beyond 120 h (data not shown) which was not observed for any of the other antimicrobials investigated. It is also not restricted to doxycycline as similar temporal increases were also observed for tetracycline and tigecycline (data not shown). This is in agreement with other studies that have shown 62 % degradation of doxycycline after 24 h (Lallemand et al., 2016). Despite the *in vitro* observations, tetracycline treatment was quite effective during the initial 1976 outbreak (Tsai et al., 1979), and there are retrospective studies of "atypical" pneumonia, including Legionnaires' disease showing that doxycycline was an effective treatment (Norrby et al., 1997; Teh et al., 2012). Experimental infection studies in guinea pigs have shown doxycycline and tigecycline are effective at preventing death from an otherwise-fatal *Legionella spp.* inoculum (Edelstein et al., 1984, 2003; Pasculle et al., 1985). Furthermore, the tetracycline family have been found to be dependent on magnesium for binding to the ribosome for inhibitory effect (Brodersen et al., 2000). As the final concentration of cations from the diluted yeast extract component would be 16.4 mg/L and 14.4 mg/L of calcium and magnesium, respectively, (very close to the specified content for CAMHB), it is unlikely that this is influencing the variability in the doxycycline MICs. Comparing MIC measurements for EUCAST *E. coli* control strain (NCTC 12241, ATCC 25922) in cation-adjusted Mueller-Hinton broth (CAMHB) relative to those achived when tested in BYE-α found doxycycline and levofloxacin MICs to be consistently one dilution lower in CAMHB but rifampicin MIC to be consistently one dilution higher relative to MIC in BYE-α. Therefore increased variation for doxycycline MICs may be influenced by insufficient magnesium, but the antimicrobial MICs for *E. coli* were still under expected ECOFF values for this susceptible quality control reference strain (data not shown).

A limitation of our study, was that we only had control strains with elevated MICs for azithromycin. The role of the LpeAB efflux pump in *Legionella* has been well established (Massip et al., 2017; Natås et al., 2019; Vandewalle-Capo et al., 2017) and our method showed a clear and signficant ($p < 0.001$) mean MIC elevation relative to the susceptible control reference strains (4.5-fold higher compared to Philadelphia-1 and 6-fold higher compared to Knoxville-1, respectively) (Fig. 4). However, judging just from our repeats it would be difficult to speculate a threshold that would capture these isolates and the maximum MIC for these isolates would likely still be below therapeutic pharmacokinetic/pharmacodynamic values achievable for *in vivo* administration. Induction of LpeAB has not been investigated and other investigators have found upto 52-fold variations in *lpeAB* expression that may impact MIC elevation (Jia et al., 2019), which may be important to the utility of identifying *lpeAB*-carrying isolates clinically. There are also some reports suggesting LpeAB only selectively elevates MICs for azithromycin, compared to the other macrolides (Minetti et al., 2024; Yang et al., 2022). However, our method easily identified the strain expected to be resistant to all macrolides carrying an A2052G mutation in three of the eight 23S *rRNA* operons, mediating an MIC up to 2048 mg/L (Ginevra et al., 2022)]. We also found a consistent and siginficant increase in lefamulin MICs to 1–4 mg/mL for this isolate, which is not unexpected given the proximity to pleuromutilin-resistance mediating hotspots from veterinary bacterial species (Pereyre and Tardy, 2021) and is consistent with the magnitude of lefamulin MIC increase reported for *Ureaplasma spp.* isolates carrying 23S *rRNA* mutations A2058G (Spiller-Boulter et al., 2021). Other limitations for this study were also intentional: restricting MIC determination to a single experienced microbiologist and restricting investigations to four strains, and no assessment of whether inclusion of amphotericin B to prolong BYE-α medium storage time altered the MIC levels for the other antimicrobials. This study was designed to establish a detailed protocol that will be further evaluated internationally to establish intra- and inter-investigator variability and refined/adjusted accordingly, as well as establish ease of use in reference laboratories of varying experience. Application of the method to clinical isolates after that phase will then establish ECOFF thresholds to define antimicrobial resistance for *L. pneumophila.*

In summary, the role of antimicrobial resistance in *L. pneumophila* (the cause of >90 % of Legionnaires' disease), cannot currently be systematically evaluted. Susceptibility testing methodologies using activated charcoal to facilitate *Legionella* growth are now deemed unreliable and clinically irrelevant by the international community. Here, we provide a charcoal-free, simple, effective, standardised alternative for antimicobial suseptibility testing that can be applied in any microbial reference laboratory equiped with a shaking incubator and define the initial ranges for five clinically relevant antimicrobials that should be able to clearly separate susceptible from putatively resistant isolates in a single plate for *L. pneumophila.* Further, we have identified four reference strains (2 susceptible and 2 with elevated macrolide MICs) to serve as quality controls, all available from NCTC or available from the corresponding author, that should be included as controls in parallel when investigating clinical isolates. This method if internationally validated could lay the foundations for future surveillance of resistance to various antimicrobials in *L. pneumophila* and potentially other *Legionella* species, as well as implementation of AST in clinical settings.

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CRediT authorship contribution statement

Max Sewell: Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Caitlin Farley:** Writing – review & editing, Supervision, Methodology, Investigation. **Edward A. R. Portal:** Writing – review & editing, Supervision, Methodology. **Diane Lindsay:** Writing – review & editing, Funding acquisition. **Maria Luisa Ricci:** Writing – review & editing, Funding acquisition. **Sophie Jarraud:** Writing – review & editing, Methodology, Funding acquisition. **Maria Scaturro:** Writing – review & editing, Validation. **Ghislaine Descours:** Writing – review & editing, Validation, Methodology. **Anne Vatland Krøvel:** Writing – review & editing, Validation. **Rachael Barton:** Writing – review & editing, Supervision, Investigation. **Ian Boostom:** Writing – review & editing, Supervision, Methodology. **Roisin Ure:** Writing – review & editing, Validation. **Darja Kese:** Writing – review & editing, Validation. **Valeria Gaia:** Writing – review & editing, Validation. **Matej Golob:** Writing – review & editing, Validation. **Susanne Paukner:** Writing – review & editing, Resources. **Christophe Ginevra:** Writing – review & editing, Validation, Resources. **Baharak Afshar:** Writing – review & editing, Validation, Resources. **Sendurann Nadarajah:** Writing – review & editing, Validation. **Ingrid Wybo:** Writing – review & editing, Validation. **Charlotte Michel:** Writing – review & editing, Validation. **Fedoua Echahdi:** Writing – review & editing, Validation. **Juana María González-Rubio:** Writing – review & editing, Validation. **Fernando González-Camacho:** Writing – review & editing,

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2024.107071.

Data availability

Data will be made available on request.

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