

## Legionella antimicrobial sensitivity testing: comparison of microbroth dilution with BCYE and LASARUS solid media

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**Objectives:** There is a lack of international unification for AST methodology for *Legionella pneumophila*. Current literature contains multiple possible methods and this study compares each of them to determine methodological concordance.

**Methods:** Antibiotic susceptibility of 50 *L. pneumophila* strains was determined using broth microdilution (BMD), serial antimicrobial dilution in traditional buffered charcoal yeast extract (BCYE) agar (as well as comparison with gradient strip overlay on BCYE) and in a novel charcoal-free agar (LASARUS) for rifampicin, azithromycin, levofloxacin and doxycycline.

**Results:** The deviation of tested media relative to BMD highlighted the overall similarity of BMD and LASARUS across all antimicrobials tested (within one serial dilution). BCYE agar dilution showed an increased MIC of up to five serial dilutions relative to BMD, while MICs by gradient strip overlay on BCYE were elevated by two to three serial dilutions, with the exception of doxycycline, which was decreased by three serial dilutions relative to MIC values determined by BMD. The MIC range for azithromycin was wider than for other antimicrobials tested and found to be caused by the presence or absence of the *lpeAB* gene.

**Conclusions:** BMD-based antimicrobial susceptibility testing (AST) methodology should be the internationally agreed gold standard for *Legionella* spp. AST, as is common for other bacterial species. Traditional BCYE gave significantly elevated MIC results and its use should be discontinued for *Legionella* spp., while MIC determination using LASARUS solid medium gave results concordant (within one serial dilution) with BMD for all antimicrobials tested. To the best of our knowledge, this study is the first to identify the *lpeAB* gene in UK isolates.

### Introduction

*Legionella pneumophila* (and to a lesser extent other *Legionella* spp.) causes Legionnaires' disease (LD), a serious community-acquired pneumonia (CAP) with a mortality rate up to 10%.<sup>1</sup> Screening for infection with *L. pneumophila* predominantly relies upon urinary antigen testing (UAT), which detects *Legionella* LPS in urine and is confirmed by culture of lower respiratory samples on media such as BCYE (buffered charcoal yeast extract) or by molecular detection methods.

Both the NICE and British Thoracic Society therapeutic guidelines recommend amoxicillin combined with a macrolide for patients with moderate-severity CAP while those with high-severity CAP should be treated with dual combination antimicrobials comprising a  $\beta$ -lactamase-stable  $\beta$ -lactam (such as co-amoxiclav) and a macrolide.<sup>2</sup> However, monotherapeutic

antimicrobial intervention is common as initial empirical therapy in patients with low-severity CAP and can include amoxicillin, clarithromycin, doxycycline and levofloxacin as the recommended first-line therapeutic agents.<sup>2,3</sup> Tetracyclines, fluoroquinolones and other macrolides have been reported as potentially effective treatments.<sup>4</sup> Identification of antibiotic resistance mechanisms in Legionellaceae is enabling detection and characterizing the emergence of reduced sensitivity in strains, demonstrated by increasing detection of the *lpeAB* macrolide efflux pump<sup>5</sup> and characterization of tetracycline deconstructase genes in *Legionella longbeachae* (more prevalent than *L. pneumophila* in CAP in Australia).<sup>6</sup> Understanding mechanisms of resistance is of particular importance for Legionellaceae wherein delay in correct effective therapy is associated with significant increase in morbidity and mortality.<sup>7</sup>

Currently, there is no international consensus on antimicrobial susceptibility testing (AST) methodology for systematic investigation of *Legionella* spp. 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 (BMD).

International standardization via CLSI, EUCAST or other reference centres requires several conditions to be met: (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); and (iv) AST to be validated by concurrent testing of universally available (ATCC-, DSMZ- and/or NCTC-deposited) 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 and IVD standards. The term 'epidemiological cut-off' (ECOFF) is defined as the *in vitro* MIC threshold that allows the discrimination of WT strains from those with acquired resistance mechanisms.<sup>8</sup> To date, there are no ECOFF values appointed for *Legionella* spp., due in part to the variation in MIC values when compared with different methodologies.

For *Legionella* spp. 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 arising from autoclaved agar. The degree of antimicrobial compound adsorption is unknown and likely to be highly variable, with the consequence of elevating the MIC.<sup>9,10</sup> 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.<sup>11</sup> Significant discrepancies between MICs determined using BMD methods and BCYE have been noted previously.<sup>9,10</sup> However, no study has performed a systematic comparison between BMD and BCYE media. This limits the currently available data for international reference by EUCAST, with current data based solely on antimicrobial gradient strips (such as Etests) on BCYE. 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.

This study describes the comparison of MICs determined for a panel of 50 *L. pneumophila* strains consisting of 35 Welsh clinical and environmental isolates, combined with 5 strains from a previously published BMD study<sup>12</sup> and 10 National Collection of Type Cultures (NCTC) control strains, comparing BMD and BCYE (antimicrobial gradient strips and dilution of antimicrobials in agar) with a novel commercially available charcoal-free *Legionella* Antimicrobial Susceptibility And Resistance Universal Screening medium (LASARUS). Here we provide a basis for future studies to

define ECOFF values to enable internationally agreed and defined resistance breakpoints for Legionellaceae.

## Materials and methods

### Reference cohort

Fifty *L. pneumophila* strains previously archived at PHE were included in the study: 22 clinical strains from Wales; 13 environmental strains from Wales; 5 previously reported strains from England,<sup>12</sup> and 10 NCTC reference strains of *L. pneumophila* (Table S1, available as [Supplementary data](#) at JAC Online).

### Antimicrobial sensitivity materials

Antimicrobials used in this study included rifampicin (Sigma–Aldrich, Poole, UK), levofloxacin hydrochloride (Sigma–Aldrich), doxycycline hydrochloride (Sigma–Aldrich) and azithromycin (Aspire Pharma, UK). All working stocks were made fresh at a concentration of 2560 mg/L in 10 mL. For rifampicin, 25.6 mg was dissolved in 1 mL of DMSO initially, prior to addition to 9 mL H<sub>2</sub>O. Doxycycline, levofloxacin and azithromycin were reconstituted initially at 100 mg/mL, as per the manufacturers' instructions, before dilution in H<sub>2</sub>O to working stocks.

The three culture media used in this study were prepared according to either the manufacturers' instructions or previously published papers: BMD<sup>5</sup>, LASARUS (Instant Test Ltd, UK) and BCYE agar and supplement (Sigma–Aldrich). All media were supplemented with 400 mg/L of L-cysteine (Sigma–Aldrich).

### Preparation of strains and antimicrobial-containing solid media

Isolates were initially revived from frozen archives by subculture onto BCYE plates and incubated in a humidified atmosphere at 37°C for 120 h. Single colonies were picked and resuspended in sterile 3 mL H<sub>2</sub>O (Thermo Fisher Scientific, USA) at a concentration equivalent to 0.5 McFarland (expected to produce an inoculum of approximately  $1.5 \times 10^8$  cfu/mL). A 0.2 mL 1:10 dilution of the 0.5 McFarland solution was made for each isolate suspension and placed in a 96-well cell culture plate (Greiner Bio-One, Germany); a new 96-well cell culture plate was made up for each tested medium and antimicrobial.

Antimicrobials were added just prior to plate-pouring into 100 mm square plates (Sarstedt, Germany) for BCYE and LASARUS solid media (one antimicrobial, one concentration per plate). For BMD, antimicrobials were mixed with buffered yeast extract broth containing 0.1%  $\alpha$ -ketoglutarate (BYE $\alpha$ )<sup>5</sup> and 0.2 mL was placed into each well of a sterile 96-well plate.

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 URI<sup>®</sup>DOT, Mast Group, UK) (Figure S1). Multipoint inoculator pins were sterilized in 80% ethanol for 10 min between ranges of a specific antimicrobial and between media types. Plates were incubated as above and read after 120 h. Isolates were tested in quadruplet.

### Antimicrobial gradient strip test

Antimicrobial gradient strips (Etest, bioMérieux, France) were overlaid on a fresh bacterial lawn (0.1 mL from a 0.5 McFarland suspension) on BCYE agar. Plates were incubated as above and read at 48, 72 and 120 h with MICs determined as the lowest concentration of antimicrobial that inhibited growth.

### Evaluation of *Legionella* growth on LASARUS

A subset of 12 NCTC strains (Table 1) were evaluated by doubling dilution (standardized by multipin inoculation on replicate plates between media types) to compare the sensitivity of *L. pneumophila* growth detection on LASARUS with that on BCYE. Solid media were inoculated with the dilution series for each strain and the highest dilution showing clear bacterial growth in a humidified incubator at 37°C after 72 and 120 h was recorded (Figure 1). Values for the highest dilution were compared by paired *t*-test analysis using GraphPad Prism (v7.03) and  $P < 0.05$  was set as the threshold for statistical significance.

### WGS

Twelve isolates showing high and 18 isolates showing low azithromycin MIC values were suspended in H<sub>2</sub>O, heat-inactivated at 95°C for 30 min and pelleted. Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Germany), with an additional RNase step, on the automated QIAcube platform (QIAGEN). Genomic DNA was quantified using the Qubit fluorometer 4.0 and the dsDNA 1X Kit (Thermo Fisher Scientific). 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).

**Table 1.** NCTC *L. pneumophila* strains used for comparison of BCYE and LASARUS growth

NCTC number	Strain designation	Serogroup
11230	Togus-1	2
11232	Bloomington-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

### Bioinformatics analysis

Bioinformatics analysis was performed using a high-performance computing cluster at Cardiff University (ARRCA) and CLIMB.<sup>13</sup> Paired-end reads (FASTQ) were subjected to quality control checks before downstream analysis. Trim Galore (v0.4.3)<sup>14</sup> 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)<sup>15</sup> and collated using MultiQC (v1.7).<sup>16</sup> 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)<sup>17</sup> and assembled into contigs using SPAdes (v3.9.0).<sup>18</sup> The trimmed FASTQ reads were mapped to the contigs using BWA (v.0.7.15)<sup>19</sup> and SAMtools (v1.3.1).<sup>20</sup> Pilon (v1.22)<sup>21</sup> was used to assess any misassemblies/errors in base-calling in the resulting mapped BAM file. All assemblies were performed within Shovill (v0.9.0). Final genome assembly metrics were generated using QUAST (v2.1).<sup>22</sup> Antibiotic resistance and plasmid genomic profiles were characterized using ABRicate and associated databases: ResFinder<sup>23</sup> and PlasmidFinder.<sup>24</sup> Genomes were identified using Pathogenwatch and annotated using Prokka (v1.12).<sup>25</sup> Further analysis was carried out using Geneious sequence analysis software (vR10; Biomatters Ltd, New Zealand). Sequences were uploaded to ENA-EMBL and assigned the accession number PRJEB40430.

### Statistical analysis

Prism GraphPad (v7.03) was used for *t*-test analysis.

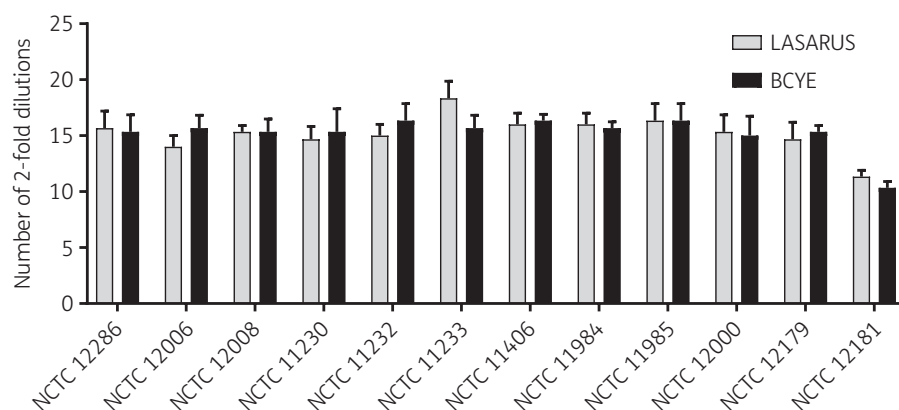
### Ethics

Ethical approval was not required. All studies were performed on archived bacterial isolates, blinded to the clinical details of the patients from which the isolates were obtained, other than that the samples had originally been referred from a Welsh hospital or a PHE collection (not relevant for NCTC reference strains).

### Results

#### Comparison of growth potential between BCYE and LASARUS agars

Prior to comparison of MIC determination, the relative capacity of the two solid media to support bacterial growth was established by the relative ability to detect growth following serial 2-fold dilution of 12 NCTC reference strains (Figure 1). LASARUS and BCYE



**Figure 1.** Growth comparison of 12 isolates in triplicate against BCYE showing similar growth patterns ( $P > 0.99$ ).

were highly comparable in their relative ability to support growth that could be easily visualized.

**Comparison of four methodologies for MIC determination: BMD, Etest (BCYE) and solid agar (LASARUS, BCYE)**

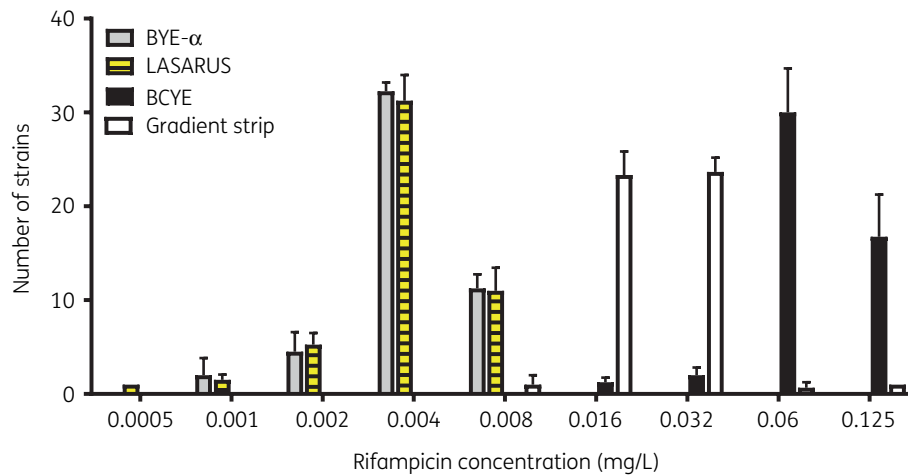
There was close concordance between liquid (BYE $\alpha$ ) medium and LASARUS agar for the determination of rifampicin MIC (Figure 2), levofloxacin MIC (Figure 3) and azithromycin MIC (Figure 4); thresholds of inhibition were found to be moderately elevated for gradient strip tests on BCYE and remarkably elevated in BCYE agar dilution.

MIC<sub>50</sub> and MIC<sub>90</sub> values for rifampicin determined on LASARUS or by BMD with BYE $\alpha$  were 0.004 and 0.008 mg/L, respectively. Values for gradient strip tests gave an MIC<sub>50</sub> of 0.032 mg/L and an

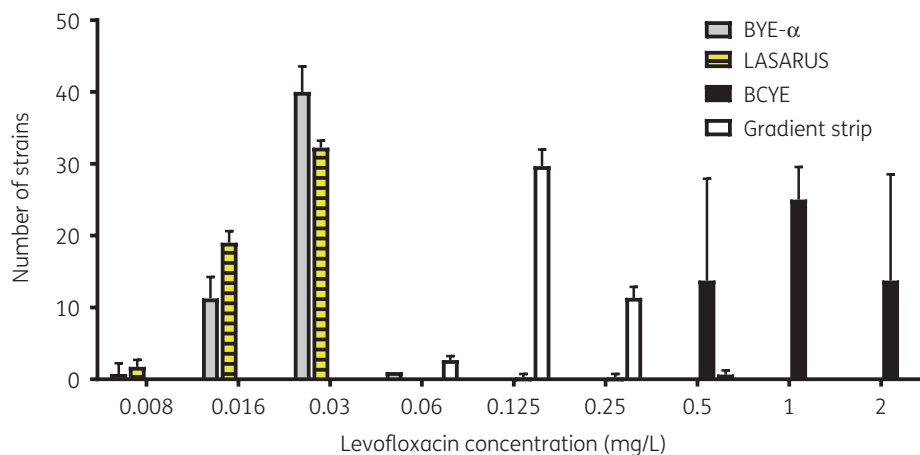
MIC<sub>90</sub> of 0.032 mg/L, while BCYE agar dilution values were elevated 16-fold to MIC<sub>50</sub> = 0.06 mg/L and MIC<sub>90</sub> = 0.128 mg/L (Figure 2).

Similarly, levofloxacin MIC values determined on LASARUS and by BMD with BYE $\alpha$  were identical, with both having MIC<sub>50</sub> = 0.03 mg/L and MIC<sub>90</sub> = 0.03 mg/L, compared with 32-fold higher values of MIC<sub>50</sub> = 1 mg/L and MIC<sub>90</sub> = 1 mg/L for BCYE, while gradient strip tests gave intermediate but still elevated values of MIC<sub>50</sub> = 0.064 mg/L and MIC<sub>90</sub> = 0.128 mg/L (Figure 3).

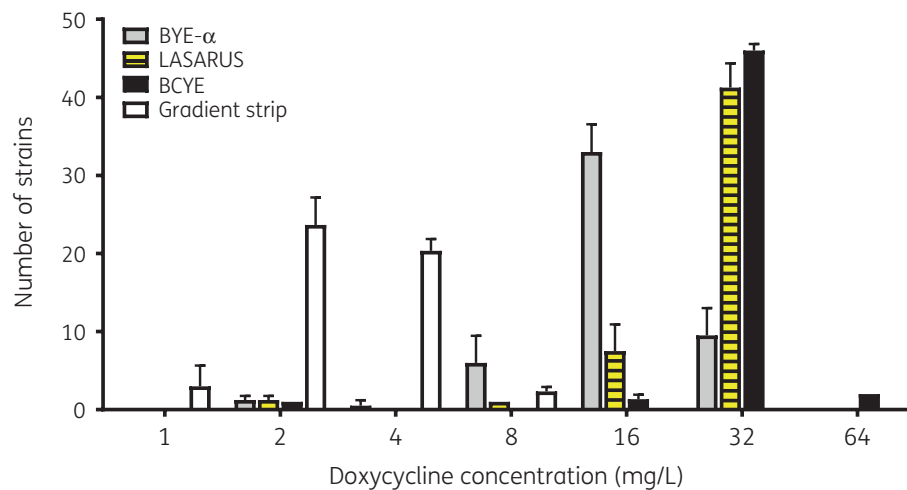
Relative to other tested antimicrobials, substantially higher concentrations of doxycycline were required to inhibit the growth of the 50 isolates of *L. pneumophila* (Figure 4) and the MICs were similar for all four methods: BYE $\alpha$  and LASARUS (MIC<sub>50</sub> = 16 mg/L and MIC<sub>90</sub> = 32 mg/L); BCYE agar (MIC<sub>50</sub> = 32 mg/L and MIC<sub>90</sub> = 32 mg/L); and reduced MIC for gradient strip tests (MIC<sub>50</sub> = 2 mg/L and MIC<sub>90</sub> = 4 mg/L).



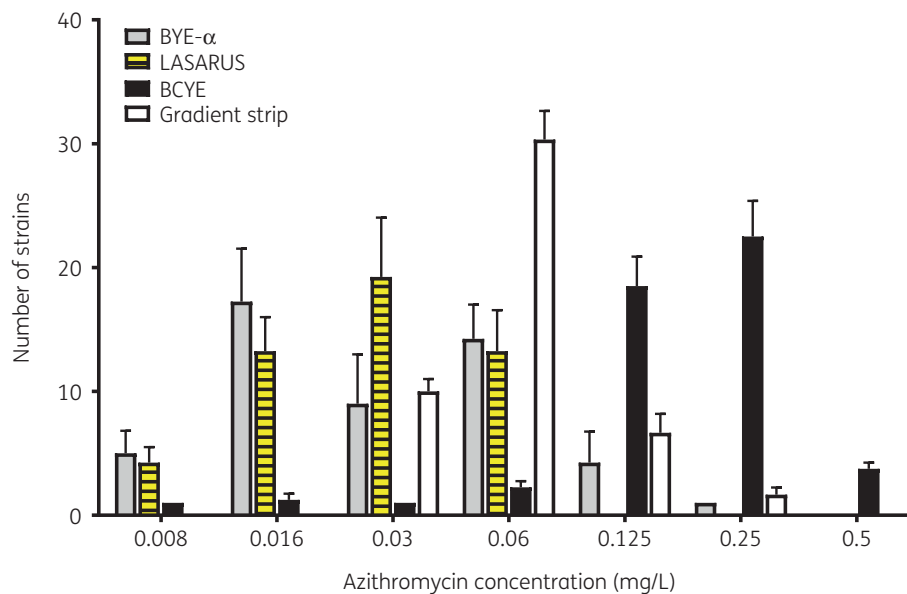
**Figure 2.** Rifampicin MIC determination for 50 *L. pneumophila*. Antimicrobials were diluted in liquid BYE $\alpha$  (grey; reference BMD method) and compared with solid media LASARUS (yellow with black stripe) and BCYE (agar dilution, black; gradient strip, white). Data represent the mean  $\pm$  SD for four replicates. Gradient strips were replicated in triplicate. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



**Figure 3.** Levofloxacin MIC determination for 50 *L. pneumophila*. Antimicrobials were diluted in liquid BYE $\alpha$  (grey; reference BMD method) and compared with solid media LASARUS (yellow with black stripe) and BCYE (agar dilution, black; gradient strip, white). Data represent the mean  $\pm$  SD for four replicates. Gradient strips were replicated in triplicate. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



**Figure 4.** Doxycycline MIC determination for 50 *L. pneumophila*. Antimicrobials were diluted in liquid BYE $\alpha$  (grey; reference BMD method) and compared with solid media LASARUS (yellow with black stripe) and BCYE (agar dilution, black; gradient strip, white). Data represent the mean  $\pm$  SD for four replicates. Gradient strips were replicated in triplicate. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



**Figure 5.** Azithromycin MIC determination for 50 *L. pneumophila*. Antimicrobials were diluted in liquid BYE $\alpha$  (grey; reference BMD method) and compared with solid media LASARUS (yellow with black stripe) and BCYE (agar dilution, black; gradient strip, white). Data represent the mean  $\pm$  SD for four replicates. Gradient strips were replicated in triplicate. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

The MIC ranges for these three antimicrobials showed little variance, generally entirely contained within a range of three to four serial dilutions for all isolates. However, a greater strain-to-strain MIC variation was observed for azithromycin (Figure 5). Overall concordant values were again observed for LASARUS and BYE $\alpha$  ( $MIC_{50}$  = 0.032 mg/L and  $MIC_{90}$  = 0.064 mg/L) relative to significantly increased BCYE MIC values ( $MIC_{50}$  = 0.256 mg/L and  $MIC_{90}$  = 0.256 mg/L) and intermediately elevated values for gradient strip tests ( $MIC_{50}$  = 0.064 mg/L and  $MIC_{90}$  = 0.128 mg/L).

#### Comparison of MIC modal deviation of LASARUS, BCYE agar dilution and gradient strip test from gold standard BMD

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

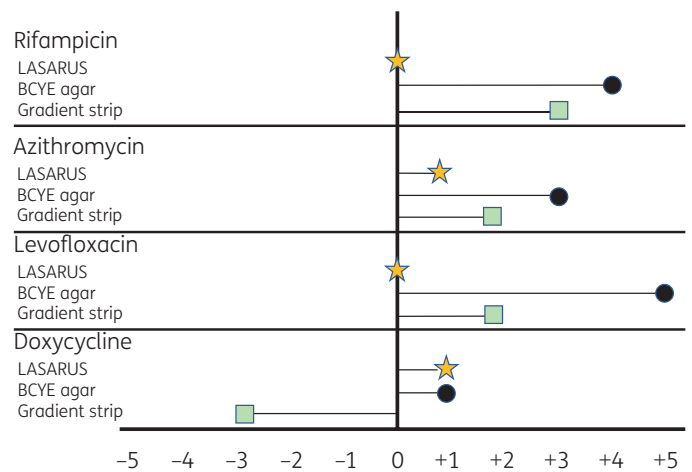
### WGS analysis of azithromycin-susceptible and -resistant strain subsets

WGS was performed on isolates at the maximal and minimal ends of the azithromycin susceptibility range to determine the presence of known mechanisms of reduced susceptibility (sequencing metrics are summarized in Table S2). Five mechanisms of macrolide antimicrobial resistance were explored by examining the WGS results for the strains of *L. pneumophila*. Macrolide resistance-mediating mutations in the 23S rRNA gene were difficult to assess due to the presence of three separate operons in all strains examined; however, no mutations or sequence heterogeneity at position 2058 or 2059 (*Escherichia coli* numbering) of the *rrl* gene 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 *erm(A)*, *erm(B)*, *erm(C)* and *erm(F)*, the macrolide phosphotransferase gene *mph(A)* and the macrolide esterase genes *ere(A)* and *ere(B)* were not observed in any of the strains. Furthermore, no presence of the efflux pump genes *mef(A/E)* was observed in any of the strains. Analysis of isolates with MIC > 0.06 mg/L using BYE $\alpha$  or LASARUS media revealed 5/10 *L. pneumophila* strains contained the *lpeAB* efflux pump gene.<sup>26</sup> Of the 18 isolates with MIC < 0.016 mg/L (tested on BYE $\alpha$  or LASARUS) that were sequenced, none contained the *lpeAB* efflux pump gene.

### Discussion

Current guidance from EUCAST recommends users should determine MICs using antimicrobial gradient strips on BCYE for Legionellaceae. ECOFF levels 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. Without standardization, this continues to confound the definition between antimicrobial resistance and sensitivity for this species.

In line with other bacterial pathogens, we utilized BYE $\alpha$  BMD as the gold standard MIC methodology, which gave concordant MICs with serial agar dilution in LASARUS. Gradient strips gave the next closest, but moderately elevated, MIC values; however, serial antimicrobial dilution in BCYE 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 (based on triplicate attempts to utilize gradient strips on *L. pneumophila* lawns grown on LASARUS). With every attempt, the zone of inhibition extended well beyond the boundaries of the strip. On LASARUS, extensive antibiotic diffusion occurred irrespective of humification of incubation. For each of these experiments, gradient strips from the same batch overlaid on *L. pneumophila*-inoculated BCYE plates, run in parallel, performed as expected. Why LASARUS has increased diffusion capacity is not clear and could be due to moisture content in the formulation promoting diffusion or another mechanism. Interestingly, the chelating effect of activated charcoal, elevating MIC determination, has been reported previously<sup>9,10,27</sup> and, while acknowledged, it has generally been disregarded as a problem in the absence of solid culture media without charcoal, despite the impact on accuracy of results. Garcia et al.<sup>11</sup> found similarly



**Figure 6.** Forest plot showing the mode of deviation of MIC when compared with gold standard BYE $\alpha$  broth. +1 equals one serial dilution above the modal average for BYE $\alpha$  BMD. LASARUS, yellow star; BCYE agar, black circle; gradient strip, green square. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

discrepant MICs when comparing antimicrobial dilution series in liquid BYE $\alpha$  with incorporation into solid BCYE.

When evaluating an individual medium, all of our isolates were inhibited within a distribution of 3- to 6-fold dilutions, similarly to previous authors,<sup>9</sup> thereby showing relatively little overall isolate variation within this dataset. However, when comparing combined data across all four methodologies in this study, the MIC distribution ranged across 6-9 serial dilutions due to the wide disparity within the four investigated methods (Figure 6). This highlights the importance of this systematic comparison, with methodologies giving widely discordant results, especially those acquired by serial agar dilution in BCYE.

Beyond the advantages of being able to perform high-throughput testing on a solid medium with multipoint inoculation, the translucent nature of LASARUS agar gave the additional advantage of safety for reading results, as opaque BCYE and 96-well plates for BMD require removal of lids to determine culture growth due to condensation. LASARUS agar would also therefore be amenable to measurement using automated optical systems, allowing increased throughput. 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, etc.). However, in practice, BMD is labour-intensive and assays requiring incubation for  $\geq 24$  h impart a much greater risk of interference by contamination relative to other bacteria that can be evaluated after 16-24 h of growth. Furthermore, the slower growth rate of 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.<sup>28</sup> showed a similar range of 0.004-0.125 mg/L, compared with our findings of 0.008-0.125 mg/L for BMD and 0.008-0.06 mg/L for LASARUS. Our MICs determined by gradient strips were moderately elevated in comparison (MIC<sub>50</sub> = 0.125 mg/L; MIC<sub>90</sub> = 0.25 mg/L), but these were similar to values determined by Sharaby et al.<sup>29</sup> using the gradient strips, with MIC<sub>50</sub> = 0.075 mg/L

and MIC<sub>90</sub> = 1 mg/L. Furthermore, while our MICs were significantly elevated for BCYE agar dilution compared with all three presented methodologies with MIC<sub>50</sub> = 1 mg/L and MIC<sub>90</sub> = 2 mg/L, these results are still similar to the results reported by Martin *et al.*<sup>30</sup> who showed MIC<sub>50</sub> = 1 mg/L for 41 *Legionella* spp. by BCYE serial dilution methods.

MIC determination for rifampicin by gradient strip tests carried out by Marques and Piedade<sup>31</sup> showed MIC<sub>50</sub> = 0.023 mg/L, concordant with the values obtained using gradient strips in this study (MIC<sub>50</sub> = 0.016 mg/L). Agar dilution on BCYE of only 30 *Legionella* strains carried out by Edelstein<sup>32</sup> showed MIC ranges between 0.03 and 0.06 mg/L, falling within those found in this study (0.016–0.125 mg/L). Reported BMD ranges for rifampicin vary from 0.00012 to 0.03 mg/L across three different publications<sup>5,12,33</sup> and Gomez-Lus *et al.*<sup>33</sup> showed MIC<sub>50</sub> = 0.004 mg/L for 140 *Legionella* spp., identical to our findings.

MICs reported for doxycycline gradient strips against 32 isolates<sup>32</sup> showed similar MIC<sub>50</sub> and MIC<sub>90</sub> values of 2 and 3 mg/L, respectively, compared with our MIC<sub>50</sub> = 2 mg/L and MIC<sub>90</sub> = 4 mg/L. The authors noted an increase in MIC during incubation on BCYE from 8 mg/L at 48 h to 32 mg/L at 72 h, highlighting potential stability of some antimicrobials leading to variation of methodology used and the importance of standardization. The unusual finding in this study was that doxycycline MICs were lower for gradient strips than all other methodologies, in contrast to findings for all other antimicrobials we tested, and this could be due to reduced dispersion of doxycycline in the media, which was previously hypothesized by Isenman *et al.*<sup>34</sup> for tetracycline.

Azithromycin MICs on BCYE by gradient strips in this study showed MIC<sub>50</sub> = 0.064 mg/L and MIC<sub>90</sub> = 0.125 mg/L, comparable to MIC<sub>50</sub> = 0.047 mg/L and MIC<sub>90</sub> = 0.25 mg/L for 100 isolates from Italy<sup>35</sup> and ranges reported by others, including 0.016–4 mg/L and 0.06–4 mg/L.<sup>5,36</sup> Further, the BMD MIC range of 0.008–0.125 mg/L in this study was similar to that reported by Sharaby *et al.*<sup>29</sup> who found an MIC range of 0.038–1 mg/L. The greater MIC range for azithromycin (relative to the tight ranges for other antimicrobials) is routinely reported and can be explained by the gene *lpeAB*.<sup>5,37</sup> Consistent with this finding, *lpeAB* was present in 5/10 *L. pneumophila* isolates with MICs above 0.06 mg/L in our study and completely absent from all sequenced strains (*n* = 18) that had MICs below 0.016 mg/L (isolate distribution consistent for both BMD and LASARUS methodologies). To the best of our knowledge, this is the first identification of this gene conferring decreased susceptibility to azithromycin in *L. pneumophila* isolates from England and Wales.

## Conclusions

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. Agreed medium formulation, incubation length and inclusion of ATCC/NCTC-deposited reference strains should be included in the international standardization of methodology.

The use of serial agar dilution using BCYE or gradient strip overlay on BCYE gave significantly elevated and more variable MIC results than gold standard BMD and therefore should be discontinued for *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 of BMD for all antimicrobials tested. Therefore, AST determination by antimicrobial agar dilution in LASARUS should be considered to have equal validity to BMD methodologies.

To the best of our knowledge, this study is the first to identify the *lpeAB* gene in isolates from England and Wales and confirmed that the 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 *Legionella* spp. makes routine MIC determination in clinical cases difficult to interpret and urgently requires international consensus.

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## Transparency declarations

None to declare. LASARUS medium has a PHE patent pending.

## Supplementary data

Tables S1 and S2 and Figure S1 are available as [Supplementary data](#) at JAC Online.

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