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Legionella antibiotic susceptibility testing: is it time for international standardization and evidence-based guidance?

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Legionella pneumophila, a Gram-negative bacillus, is the causative agent of Legionnaire's disease, a form of severe community-acquired pneumonia. Infection can have high morbidity, with a high proportion of patients requiring ICU admission, and up to 10% mortality, which is exacerbated by the lack of efficacy of typical empirical antibiotic therapy against *Legionella* spp. The fastidious nature of the entire Legionellaceae family historically required inclusion of activated charcoal in the solid medium to remove growth inhibitors, which inherently interferes with accurate antimicrobial susceptibility determination, an acknowledged methodological shortfall, now rectified by a new solid medium that gives results comparable to those of microbroth dilution. Here, as an international *Legionella* community (with authors representing various international reference laboratories, countries and clinical stakeholders for diagnosis and treatment of legionellosis), we set out recommendations for the standardization of antimicrobial susceptibility testing methods, guidelines and reference strains to facilitate an improved era of antibiotic resistance determination.

There are over 60 species within the genus *Legionella*, the majority of which have been isolated only from environmental, rather than from clinical, sources. These Gram-negative bacteria are naturally found in water and soil, are thought to propagate within amoebae and become particularly problematic when they contaminate domestic water systems, spas, pools and cooling towers. It was contamination of an air conditioning unit at the Philadelphia Bellevue-Stratford Hotel in 1976 that is the suspected source of the initial "Legionnaires' disease" that resulted in the hospitalization of 182 people, with the subsequent death of 29; most within a week of the American Legion conference, after which the bacteria are named. Over 90% of cases of severe community-acquired pneumonia arising from legionellosis are attributed to the species *Legionella pneumophila* and further subclassification shows serogroup 1 is dominant with regard to disease association; however, most testing methodologies are focussed towards

L. pneumophila serogroup 1 detection. Currently, legionellosis is found as large outbreaks, in addition to sporadic infections requiring hospital admission that are largely community-associated, travel-acquired or nosocomial. During pneumonia, *Legionella* spp. invade and replicate within pulmonary macrophages, which limits effective therapeutics to those with intracellular penetration (e.g. fluoroquinolones, macrolides, streptogramins, rifampicin, pleuromutilins, tetracyclines and trimethoprim)[1] and excludes those with poor penetration (e.g. aminoglycosides, penicillins and cephalosporins).[1] The proportion of patients with legionellosis who do not receive antibiotics in the community or are given empirical therapy for pneumonia that fails to resolve the infection is not known and may impact the number of hospital admission for legionellosis, especially when the MICs of tetracyclines,[2] which are a common choice for treatment in the community, appear to be elevated when tested *in vitro*. Given the significant limitation of antibiotics available to treat legionellosis, it is particularly important to be able to identify emerging resistance to those therapeutics that remain. For less common human-infecting *Legionella* species there is a paucity of data on the correlation between *in vitro* susceptibility levels and clinical effectiveness and a lack of data on the intracellular location of these species during infection. Further studies are needed to improve understanding of these factors in legionellosis, especially as an increase in non-*L. pneumophila* infection case detection with increased molecular diagnostics is likely.

International guidelines, epidemiological cut-off values, well-validated methodologies and control strains validated in multiple laboratories are all absent for antimicrobial susceptibility testing of *Legionella*. Examination of the existing reports that are described in the literature for Legionellaceae (Table S1, available as Supplementary data at *JAC* Online) shows a wide variation in MIC values and methods utilized for susceptibility testing. Currently, gradient MIC strip testing on buffered charcoal-containing yeast extract (BCYE) agar is the methodology recommended by EUCAST.[3] There are a number of technical caveats with this method, including the sequestration of antibiotics by the activated charcoal present in the medium, which accounts for the documented rise in the MIC results, as well as variations in charcoal content from plate-to-plate due to suspension settling during pouring.[4,5] Recent work by Portal *et al.*[2] (this issue) demonstrated that gradient strip testing and BCYE agar dilution methodologies gave higher MIC values than the microbroth dilution method used in Vandewalle-Capo *et al.*[6] (2017). Portal *et al.*[2] describe the use of a charcoal-free solid medium for *Legionella* that generated concordant MIC values when compared with those determined using the broth microdilution method. This finding provides a reliable solid medium alternative to microbroth dilution for testing *Legionella* susceptibility, which is also more applicable on a routine basis. However, it is important to note that *in vitro* testing is only the first step in the eventual goal of determining clinical resistance thresholds that take into account *in vivo* drug pharmacokinetics and pharmacodynamics, especially as *in vitro* antibiotic susceptibility testing does not take into consideration cellular penetration; important as *L. pneumophila* is known to infect macrophages. An example of the importance of *in vivo* testing is demonstrated by the unexpected efficacy of doxycycline, which despite having an elevated *in vitro* MIC (BCYE agar MIC = 56 mg/L or BYE broth MIC = 1 mg/L) was as effective as erythromycin against *L. pneumophila* in an animal model study,[7] and tigecycline (BYE broth MIC = 4) was as effective as azithromycin for preventing death in a Legionnaire's disease animal model study.[8]

International treatment recommendations for patients with *Legionella* infection are also inconsistent, often providing differing guidelines and regimens[9–11] 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[12] and single point somatic mutations in *L. pneumophila* 23S rRNA[13] have increased, which mediate intermediate to high levels of macrolide resistance. Moreover, a recently documented novel tetracycline resistance gene in *Legionella longbeachae*[14] (the most common source of legionellosis in Australia and New Zealand), which may be on a mobile genetic element, highlights the need for standardization and validation at an international level. 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 culture and because some patients are culture negative. However, enabling evidence-based treatment guidance internationally and nationally is required and for patients with persistent infection or for water systems known to be colonized with *Legionella* that pose ongoing risks for patients (such as contaminated hospital systems) understanding the MIC may be of clinical benefit. This is confounded by the unknown significance of a substantial increase 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.

Due to the lack of comparable data and the varied approaches and methodologies in use across the globe to address this topic, the international *Legionella* community (which includes representation from many international *Legionella* reference laboratories, as well as from the EUCAST Steering Committee and the CDC) have agreed the following recommendations:

(i) Gradient strip testing on BCYE agar should be discontinued as the recommended EUCAST methodology, due to higher (and more variable) MIC results when compared with microbroth dilution.
(ii) BCYE agar should no longer be used for serial antibiotic dilution MIC determination for *Legionella* due to higher MIC results and antibiotic sequestration.
(iii) Future studies should develop and standardize microbroth dilution 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).
(iv) The *Legionella* community is in the process of identifying and validating a panel of clinical and environmental reference strains for MIC determination. Three leading candidate strains being evaluated in a multicentre site study include: (a) *L. pneumophila* antibiotic-susceptible strain W872, serogroup 1, monoclonal subgroup Benidorm (culture collections: NCTC 12821, DSM 27564, CCUG 67715, WDCM 00205); (b) *L. pneumophila* antibiotic-susceptible strain Philadelphia 1, serogroup 1, monoclonal subgroup Philadelphia (culture collections: ATCC 33152 NCTC 11192, CCUG 9568, CIP 103854, DMZ 7513, JCM 7571, WDCM 00107); and (c)

L. pneumophila LpeAB macrolide efflux pump-containing strain Paris, serogroup 1, monoclonal subgroup Philadelphia (culture collection: CIP 107629).

(v) The *Legionella* community will endeavour to develop a consensus standard operating procedure, define epidemiological cut-off values and develop consensus on antibiotic testing of strains of clinical relevance. This should include rapidly growing bacterium controls to determine if a particular antimicrobial agent is inactivated by the test medium, such as *Escherichia coli* (ATCC 25922 NCTC 12241) or *Staphylococcus aureus* (ATCC 25923 NCTC 12981).

(vi) Antimicrobial susceptibility testing for *Legionella* using a recognized gold-standard methodology will be encouraged as part of a global surveillance for the emergence of resistance.

(vii) Phenotypically resistant *Legionella* strains should be comprehensively analysed using WGS and other complementary methods in order to identify new and emerging mechanisms underlying resistance in Legionellaceae.

To the best of our knowledge, this is the first international viewpoint on antibiotic resistance in *Legionella*. It is hoped this viewpoint will spark improved international consensus and quality in microbiological antibiotic susceptibility testing, impacting improved understanding of the level and mechanisms of resistance to antibiotics in *Legionella* and ultimately providing more accurate data used internationally to inform clinicians treating those infected with *Legionella*.

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Disclaimer

The views expressed are those of the authors and not necessarily those of their respective institutions and health providers. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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