Journal of Bioterrorism & Biodefense
ISSN: 2157-2526
Research Article Open Access

An Alternative MIC Method to Evaluate the *In vitro* Activity of Beta-Lactam Antibiotics against *Francisella tularensis*

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Francisella tularensis, the causative agent of the disease tularemia, is a Gram-negative bacterium which can be transmitted to humans through the skin, via the oral route or as an aerosol. Infections in humans can be severe and thus the pathogen is classified by the CDC as a category A bacterial agent. Early treatment with an aminoglycoside, a fluoroquinolone or doxycycline, reduces the mortality associated with *F. tularensis* infection to approximately 1-2.5% [**1**], however there are reports of therapeutic-resistant strains emerging, warranting the investigation of new antibiotics. *F. tularensis* strain SchuS4 is a well characterized and routinely utilized Type A strain, capable of causing serious infection, particularly by the inhalational route, and has an infectious dose of <10 CFU [**2**]. Although highly infectious, *F. tularensis* is slow to culture within the laboratory in liquid media grown statically, particularly in microliter volumes in microtitre plates (the standard methodology for minimum inhibitory concentration (MIC) determination of antibiotics). The addition of supplements (eg cysteine) to the growth media and extended incubation (48 hours) is required to achieve growth.

Ceftobiprole is an advanced generation cephalosporin that has demonstrated *in vitro* activity and *in vivo* efficacy against clinically important Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* [**3**]. Ceftobiprole has previously demonstrated *in vitro* activity against a panel of *F. tularensis* strains grown in Cation Adjusted Muller Hinton Broth (CAMHB) supplemented with 2% Isovitalex (Becton Dickinson) [**4**], however, since MICs for the QC strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 were unexpectedly high [**4**], under these growth conditions, it was hypothesized that the *in vitro* MICs for the *F. tularensis* strains were also high due to inhibition of ceftobiprole activity by Isovitalex or degradation following extended incubation at 37°C. Differences in MIC by different assays have been reported previously for oximino-type expanded-spectrum cephems and *Proteus vulgaris*, where MICs were classed as resistant by the broth microdilution method but susceptible by the agar dilution MIC assay [**5**].

Ceftobiprole MICs determined at Dstl with *F. tularensis* strain SchuS4 using Modified Cysteine Partial Hydrolysate Broth (MCPH) supplemented with 4% glucose and 100 µg/mL of cysteine in the broth microdilution assay were not reproducible, with ceftobiprole MICs ranging from 0.03-32 µg/mL. It was unclear whether this variability was due to antagonism between the cysteine supplement and ceftobiprole, the degradation of ceftobiprole over the incubation period of the assay or a combination of both factors [**6**]. The aim of this work was to investigate the use of the broth macrodilution MIC assay with and without supplemental cysteine as an alternative method to evaluate ceftobiprole against *F. tularensis* strain SchuS4. The macrodilution assay allows for the culturing of this fastidious organism in conditions previously proven to improve growth (e.g. larger broth volumes and shaking). Ciprofloxacin was included as a comparator antibiotic and positive control and QC strain *S. aureus* ATCC 29213 was also evaluated.

A 10 mg/mL stock of ceftobiprole was prepared using 1% acetic acid and 99% dimethyl sulphoxide and vortexing for 15 mins. A 10 mg/mL stock of ciprofloxacin was prepared by adding 9 mL of distilled water to 100 mg of antibiotic followed by the addition of 1 mL of 1M sodium hydroxide. The MICs for ceftobiprole and ciprofloxacin were determined in MCPH supplemented with 4% glucose, with or without the addition of 100 µg/mL of cysteine (*F. tularensis*) or CAMHB (*S. aureus*). Bacteria were inoculated at a final concentration of approximately 5 x 10^5 CFU/mL (*S. aureus*) or 1 x 10^6 CFU/mL (*F. tularensis*) in a 10ml total volume. The bacterial cultures were incubated for 24 hours (*S. aureus*) or 48 hours (*F. tularensis*), at 37°C and read by eye. All assays were performed in triplicate within a Containment Level 3 laboratory. Positive growth controls (without antibiotics, with or without cysteine or cultured with the diluents) were included.

The controls all grew as expected. The ceftobiprole and ciprofloxacin MICs for *S. aureus* with and without cysteine were in range (0.12-1 µg/mL and 0.12-0.5 µg/mL for ceftobiprole and ciprofloxacin respectively). Similarly the ciprofloxacin MICs for *F. tularensis* with or without cysteine were in the range of 0.015-0.03 µg/mL, typically reported for the antibiotic and pathogen. The ceftobiprole MICs at 48 hours grown in media without cysteine were consistently 0.03-0.06 µg/mL. Therefore, it is hypothesized that the cysteine in the growth media had an inhibitory effect on the ceftobiprole MICs in the broth microdilution assay with *F. tularensis*.

Conclusion

In summary, the broth macrodilution culture conditions without the cysteine supplement resulted in sufficient *F. tularensis* growth to accurately determine the ceftobiprole MICs, which were reproducible in multiple assays. This alternative method can be utilized to determine the ceftobiprole MICs for additional *F. tularensis* strains and allows for further development including pharmacokinetic/pharmacodynamics studies to be performed, to define the dose required for *in vivo* efficacy studies. This alternative methodology may be explored with other compounds where cysteine supplementation is hypothesized to interfere with activity.

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Received: 20-Jan-2023, Manuscript No: jbtbd-23-87701, **Editor assigned:** 23-Jan-2023, PreQC No: jbtbd-23-87701 (PQ), **Reviewed:** 27-Jan-2023, QC No: jbtbd-23-87701, **Revised:** 30-Jan-2023, Manuscript No: jbtbd-23-87701 (R) **Published:** 31-Jan-2023, DOI: 10.4172/2157-2526.1000322

Citation: Richards MI, Barnes KB, Smart JI, Jones M, Harding SV (2023) An Alternative MIC Method to Evaluate the *In vitro* Activity of Beta-Lactam Antibiotics against *Francisella tularensis*. J Bioterr Biodef, 14: 322.

Funding

This project was funded in part with federal funds from the Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response, Biomedical Advanced Research and Development Authority, under contract no. HHSO100201600002C, and in part by Basilea Pharmaceutica International Ltd., Allschwil, Switzerland.

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