

In Vitro Susceptibility of Isolates of *Francisella tularensis* Types A and B from North America[∇]

Sandra K. Urich and Jeannine M. Petersen*

Bacterial Diseases Branch, Division of Vector-Borne Infectious Diseases, National Center for Zoonotic, Vector-Borne, and Enteric Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado

Received 7 December 2007/Returned for modification 9 March 2008/Accepted 5 April 2008

Due to concern that *Francisella tularensis*, the causative agent of tularemia, may be used as a bioterrorist weapon, the Clinical and Laboratory Standards Institute recently provided a susceptibility testing method with breakpoints. Here, 169 isolates (92 type A and 77 type B) from North America were tested against seven antimicrobial agents (streptomycin, gentamicin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, and chloramphenicol) used for the treatment of tularemia. The MICs for all of the isolates fell within the susceptible range. In addition, all isolates had MICs for erythromycin of 0.5 to 4 µg/ml, in contrast to an MIC of >256 µg/ml for the common laboratory strain LVS (live vaccine strain).

Francisella tularensis is the causative agent of tularemia, a zoonosis of the northern hemisphere. In recent years, interest in this bacterium has been heightened because of concern that it may be used as a bioterrorist weapon (4, 9). Humans contract tularemia from rabbits and rodents, biting insects and ticks, and occasionally by inhalation of infectious aerosols. Cases in the United States are sporadic or occur in small clusters (4). In North America, two subspecies, *F. tularensis* subsp. *tularensis* (type A) and *holarctica* (type B), cause disease. Type A has been further subdivided into two subpopulations, A1 and A2, which differ with respect to geographic location and clinical illness in infected humans (11).

F. tularensis has been reported to be susceptible to a variety of antimicrobial agents, including aminoglycosides, chloramphenicol, quinolones, and tetracyclines (12). Streptomycin was established early as the drug of choice for treating tularemia. Treatment with another aminoglycoside, gentamicin, has been more common in more recent years. Bacteriostatic agents such as chloramphenicol and tetracyclines have also been used but are associated with a higher risk of relapse. Quinolones, which have intracellular activity, have been introduced most recently as possible options for treatment.

The purpose of this study was to evaluate a large panel of geographically and temporally diverse *F. tularensis* isolates from North America against traditional and newer antimicrobial agents. Although several reports assessing antimicrobial susceptibility have been conducted (1, 5, 6, 7, 8, 10, 13), much of these data were not obtained by a Clinical and Laboratory Standards Institute (CLSI)-approved susceptibility method, raising the possibility that resistance went undetected. Recently, CLSI provided a method and breakpoints for antimicrobial susceptibility testing of *F. tularensis* (2). In this study, 169 *F. tularensis* strains (92 type A and 77 type B) from North

America were tested by the CLSI-recommended broth microdilution procedure for *F. tularensis* (2, 3).

F. tularensis strains were submitted to the Centers for Disease Control and Prevention from 40 U.S. states and Canada between 1974 and 2005 and maintained at -75°C in brain heart infusion broth with 10% glycerol. The sources of these isolates were humans ($n = 143$) and animals (rabbits, rodents, and primates; $n = 26$). Isolates were confirmed as *F. tularensis* by characteristic growth on cysteine heart agar with 9% chocolate sheep blood (CHAB) and direct fluorescent-antibody staining. The subspecies (types A and B) were differentiated by glycerol fermentation with a GN2 microplate and the MicroLog System (Biolog, Inc., Hayward, CA). Type A strains were divided into two subpopulations, A1 and A2, based on PmeI pulsed-field gel electrophoresis subtyping (11). All work with *F. tularensis* cultures was performed in a biosafety level 3 laboratory with biosafety level 3 safety precautions.

Seven antimicrobial agents with doubling dilutions in their therapeutic ranges were tested: 0.03 to 64 µg/ml for gentamicin, 0.25 to 512 µg/ml for streptomycin, 0.06 to 128 µg/ml for tetracycline, 0.03 to 64 µg/ml for doxycycline, 0.001 to 2 µg/ml for ciprofloxacin, 0.004 to 8 µg/ml for levofloxacin, and 0.12 to 256 µg/ml for chloramphenicol. Erythromycin (0.5 to 256 µg/ml) was added to assess whether any North American strains are resistant to this drug since the MIC of erythromycin is in the resistant range for type B strains in northern Europe and Russia (6, 8, 12, 13). There are no CLSI guidelines for erythromycin with gram-negative organisms, so breakpoints for resistance are unavailable. However, erythromycin sensitivity could aid in identifying *F. tularensis* subsp. *holarctica* (type B) isolates from outside North America. Therefore, *F. tularensis* subsp. *holarctica* LVS was included as a control in this study since it originates from Russia and is known to be erythromycin resistant.

Broth microdilution plates were prepared with the CLSI-recommended media for *F. tularensis* (cation-adjusted Mueller Hinton broth supplemented with 2% defined growth supplement [IsoVitaleX], pH 7.3 ± 1) by Trek Diagnostic Systems, Cleveland, OH. Growth and purity control wells were included on all plates. Plates arrived frozen and were stored at -75°C

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases, Foothills Campus, 3150 Rampart Road, Ft. Collins, CO 80521. Phone: (970) 266-3524. Fax: (970) 494-6631. E-mail: nzp0@cdc.gov.

[∇] Published ahead of print on 14 April 2008.

TABLE 1. Antimicrobial MIC distributions for 169 *F. tularensis* isolates

Antimicrobial	MIC (µg/ml) ^a																				
	0.001	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	
Chloramphenicol	NT ^b	NT	NT	NT	NT	NT	NT			8	88	67	6							NT	
Ciprofloxacin			1	3	69	87	9						NT	NT	NT	NT	NT	NT	NT	NT	
Doxycycline	NT	NT	NT	NT	NT				20	50	73	21	5							NT	NT
Gentamicin	NT	NT	NT	NT	NT	23	72	55	16	3										NT	NT
Levofloxacin	NT	NT			12	103	50	4							NT	NT	NT	NT	NT	NT	
Streptomycin	NT	NT	NT	NT	NT	NT	NT	NT	9	23	82	47	8								
Tetracycline	NT	NT	NT	NT	NT	NT		2	26	75	58	8								NT	NT

^a Vertical bars indicate susceptibility breakpoints.
^b NT, not tested for susceptibility to that concentration.

until use. Prior to broth microdilution susceptibility testing, *F. tularensis* isolates were subcultured from frozen stocks onto CHAB, followed by two additional subcultures on chocolate agar II plates (BD Diagnostic Systems, Sparks, MD) for 48 h at 35°C. Quality control strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29513, and *Pseudomonas aeruginosa* ATCC 27853, stored at -75°C, were subcultured three times to sheep blood agar for 18 to 24 h at 35°C. Inocula were prepared by suspending colonies in Mueller-Hinton broth (BD Diagnostic Systems) to a 0.5 McFarland standard with a turbidity meter. This suspension was diluted 20-fold, and 10 µl was inoculated with broth microdilution inoculators (PML Microbiologicals, Wilsonville, OR) into freshly thawed broth microdilution plates at room temperature. Plates were covered with adhesive covers, placed into plastic bags, and incubated in ambient air at 35°C. The final inoculum concentrations determined by colony counts from the growth control well were ~5 × 10⁵ CFU/ml and >2 × 10⁶ CFU/ml for the quality control and *F. tularensis* strains, respectively. The MICs were read at 24 and 48 h for quality control strains and at 48 h for *F. tularensis* isolates (2). Antimicrobial testing with the quality control strains was performed with every batch of *F. tularensis* isolates to verify that the results fell within the acceptable range (2).

The MIC distributions for the 169 isolates are listed in Table 1. For the seven antimicrobial agents tested, the MICs fell within the susceptible range for *F. tularensis* defined in the CLSI standards (2). The MICs that inhibited the growth of 50 and 90% of the isolates (MIC₅₀ and MIC₉₀, respectively) are shown in Table 2. The MIC₅₀s and MIC₉₀s for the two *F. tularensis* subspecies, type A and type B, were in agreement, with variation within 1 doubling dilution, for all seven antibiotics. The MIC₉₀s for the A1 (n = 65) and A2 (n = 23) strains were also in agreement, with variation within 1 doubling dilution, for all seven antibiotics. Overall, the most active antimicrobial agents in vitro were the fluoroquinolones.

Regarding erythromycin, all 169 North American strains (*F. tularensis* types A and B) fell within the MIC range of 0.5 to 4 µg/ml. The MIC₅₀ and MIC₉₀ for type A and type B strains differed by 2 doubling dilutions, with lower MICs for type A strains. In contrast to the North American strains, for the Russian *F. tularensis* type B strain (LVS), the MIC of erythromycin was >256 µg/ml (not shown). Thus, erythromycin sensitivity differs between isolates from North America and LVS, an attenuated strain common in many academic and public health laboratories throughout North America.

Although previous studies have examined the susceptibilities of *F. tularensis* isolates to various antimicrobial agents, no

TABLE 2. In vitro activities of eight antibiotics against 169 isolates of *F. tularensis*^a

Antimicrobial	Type B strains			Type A strains		
	MIC range	MIC ₅₀	MIC ₉₀	MIC range	MIC ₅₀	MIC ₉₀
Aminoglycosides						
Gentamicin	0.03–0.5	0.12	0.25	0.03–0.25	0.06	0.12
Streptomycin	0.25–4	1	2	0.25–4	1	2
Tetracyclines						
Tetracycline	0.25–2	0.5	1	0.12–2	0.5	1
Doxycycline	0.25–4	1	2	0.25–2	0.5	1
Fluoroquinolones						
Ciprofloxacin	0.004–0.06	0.03	0.06	0.008–0.06	0.015	0.03
Levofloxacin	0.015–0.12	0.03	0.06	0.015–0.12	0.03	0.06
Phenolics						
Chloramphenicol	0.5–4	2	2	0.5–2	1	2
Other						
Erythromycin	0.5–4	2	2	0.5–2	0.5	0.5

^a Values are in micrograms per milliliter.

standardized method of testing or interpretative criteria had been established. In 2006, CLSI provided a broth microdilution method for *F. tularensis* with breakpoints including interpretive criteria for quality control organisms. With this standardized methodology, no antimicrobial resistance to seven antimicrobial agents (streptomycin, gentamicin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, and chloramphenicol) used for treatment was detected in North American strains. These results are consistent with other antimicrobial studies performed with *F. tularensis* isolates from throughout the northern hemisphere and the fact that treatment failure due to resistance of *F. tularensis* to the antibiotics used for clinical therapy has never been demonstrated (1, 5, 6, 7, 8, 10, 12, 13).

We acknowledge Kristy Kubota, Aimee Janusz, and Kiersten Kugeler for their assistance in performing pulsed-field gel electrophoresis analysis on type A strains in this study and Marty Schriefer for critical review of the manuscript.

We also acknowledge all of the state and local health departments that obtained isolates used in this study.

REFERENCES

1. Baker, C. N., D. G. Hollis, and C. Thornsberry. 1985. Antimicrobial susceptibility testing of *Francisella tularensis* with a modified Mueller-Hinton broth. *J. Clin. Microbiol.* **22**:212–215.
2. Clinical and Laboratory Standards Institute. 2006. Performance standards for antimicrobial susceptibility testing. Sixteenth informational supplement. CLSI document M100-S16. Clinical and Laboratory Standards Institute, Wayne, PA.
3. Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard—seventh edition. CLSI document M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
4. Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, K. Tonat, and the Working Group on Biodefense. 2001. Tularemia as a biological weapon: medical and public health management. *JAMA* **285**:2763–2773.
5. García del Blanco, N., C. B. Gutiérrez Martín, V. A. de la Puente Redondo, and E. F. Rodríguez Ferri. 2004. *In vitro* susceptibility of field isolates of *Francisella tularensis* subsp. *holarctica* recovered in Spain to several antimicrobial agents. *Res. Vet. Sci.* **76**:195–198.
6. Ikäheimo, I., H. Syrjälä, J. Karhukorpi, R. Schildt, and M. Koskela. 2000. *In vitro* antibiotic susceptibility of *Francisella tularensis* isolated from humans and animals. *J. Antimicrob. Chemother.* **46**:287–290.
7. Johansson, A., S. K. Urich, M. C. Chu, A. Sjöstedt, and A. Tärnvik. 2002. *In vitro* susceptibility to quinolones of *Francisella tularensis* subspecies *tularensis*. *Scand. J. Infect. Dis.* **34**:327–330.
8. Kudelina, R. I., and N. G. Olsufiev. 1980. Sensitivity to macrolide antibiotics and lincomycin in *Francisella tularensis holarctica*. *J. Hyg. Epidemiol. Microbiol. Immunol.* **24**:84–91.
9. Petersen, J. M., and M. E. Schriefer. 2005. Tularemia: emergence/re-emergence. *Vet. Res.* **36**:455–467.
10. Scheel, O., T. Hoel, T. Sandvik, and B. P. Berdal. 1993. Susceptibility pattern of Scandinavian *Francisella tularensis* isolates with regard to oral and parenteral antimicrobial agents. *APMIS* **101**:33–36.
11. Staples, J. E., K. A. Kubota, L. G. Chalcraft, P. S. Mead, and J. M. Petersen. 2006. Epidemiologic and molecular analysis of human tularemia, United States, 1964–2004. *Emerg. Infect. Dis.* **12**:1113–1118.
12. Tärnvik, A., and M. C. Chu. 2007. New approaches to diagnosis and therapy of tularemia. *Ann. N. Y. Acad. Sci.* **1105**:378–404.
13. Tomaso, H., S. Al Dahouk, E. Hoefler, W. Spletstoeser, T. Treu, M. Dierich, and H. Neubauer. 2005. Antimicrobial susceptibilities of Austrian *Francisella tularensis holarctica* biovar II strains. *Int. J. Antimicrob. Agents* **26**:279–284.