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In Vitro Antibiotic Susceptibilities of *Yersinia pestis* Determined by Broth Microdilution following CLSI Methods

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***In vitro* susceptibilities to 45 antibiotics were determined for 30 genetically and geographically diverse strains of *Yersinia pestis* by the broth microdilution method at two temperatures, 28°C and 35°C, following Clinical and Laboratory Standards Institute (CLSI) methods. The *Y. pestis* strains demonstrated susceptibility to aminoglycosides, quinolones, tetracyclines, β-lactams, cephalosporins, and carbapenems. Only a 1-well shift was observed for the majority of antibiotics between the two temperatures. Establishing and comparing antibiotic susceptibilities of a diverse but specific set of *Y. pestis* strains by standardized methods and establishing population ranges and MIC_{50} and MIC_{90} values provide reference information for assessing new antibiotic agents and also provide a baseline for use in monitoring any future emergence of resistance.**

Yersinia pestis is the causative agent of plague, a rare infection in humans that usually appears in the bubonic form due to flea bites. Pneumonic plague is an infection of the respiratory tract which can be contagious and rapidly fatal. This form of plague has an incubation period of 3 to 5 days and a mortality rate near 100% (1, 2). Antibiotic intervention can offer relief but only if started very early in the infection (1). Because of the scattered information on MICs under a variety of nonstandardized testing conditions (3–6) and the lack of comparative data on type strains, we report specific antibiotic susceptibility results from use of the Clinical and Laboratory Standards Institute (CLSI) microdilution broth methodology for 30 strains of *Y. pestis*. This information will be highly useful as baseline data in the event of wartime or terrorist release and for natural and laboratory-acquired infections.

TABLE 1 *Y. pestis* strain information

Y. pestis strain	Biovar/genotype/isotype[a]	Geographic origin
Colorado 92	Ort/A/O1	USA
C12	Ort/A/O1	USA
Antiqua	Ort/A/A3	Congo
Pestoides B	Med/D/P1	FSU[b]
Pestoides F	Ant/K/P2	FSU
Yeo154	Ant/F/A1a	Japan
Angola	Ant/J/A4	Angola
Java9	Ort/A/O1	Indonesia
M111(74)	Ort/B/O1	Madagascar
LaPaz	Ort/A/O1	Bolivia
195P	Ort/A/O1	India
T26	Ant/–/–	Tanzania
KIM 10	Med/I/M1a	Kurdistan
Pestoides E	Ant/M/P2	FSU
RFPBM 19	Ort/A/O2a	Burma
PEXU 429	Ort/A/O1	Brazil
Yokohama	Ant/F/A1a	Japan
Nicholisk 41	Med/G/M2	Manchuria
Nairobi	Ant/F/A2	Kenya
South Park	Ort/A/O1	USA
Cambodia	Ort/A/–	Cambodia
27	Ort/–/O2b	Vietnam
31	Ort/–/O2a	Vietnam
390	Ort/–/O1	Israel
590	Ort/B/O1	Brazil
25	Ort/–/O2c	Vietnam
316	–/A/O2a	Unknown
366	Med/H/M1b	Yemen
Harbin 35	Med/E/M2	Manchuria
Pestoides C	Med/E/P1	FSU

[a] Biovars and genotypes were taken from reference 7, and isotypes were taken from reference 8.
[b] FSU, former Soviet Union.

MATERIALS AND METHODS

The *Y. pestis* strains used in this study (shown in Table 1) were obtained from the USAMRIID collection and selected to represent established biovars, genotypes, and isotypes (7, 8). Most of the antibiotics were obtained from the U.S. Pharmacopoeia (Rockville, MD) except for ceftriaxone and fusidic acid (Sigma Chemical Co., St. Louis, Mo), cethromycin (Advanced Life Sciences), telithromycin (Sanofi-Aventis), garenoxacin (Schering-Plough), gemifloxacin (Oscient), ertapenem (Merck), faropenem (Replidyne), and tigecycline (Wyeth). Most of the stock solutions (5 mg/ml) were prepared for each drug in the appropriate solvents, based on the current CLSI recommendations (9), and stored until use at −70°C. The concentration of our amoxicillin-clavulanate (2:1) stock was 5 mg/2.5 mg per ml, and that of the co-trimoxazole stock was 5 mg/ml sulfamethoxazole and 0.26 mg/ml trimethoprim (19:1). The MICs were determined by the microdilution method in 96-well plates as previously described (10). Antibiotics were serially diluted 2-fold in 50 μl of cation-adjusted Mueller-Hinton broth (CAMHB). The antibiotic range in the plates was 64 to 0.008 μg/ml based on a final well volume of 100 μl after inoculation. The inocula were prepared by picking several colonies from sheep blood agar (SBA) plates grown for 36 to 48 h at 28°C, suspended, and diluted with CAMHB to a bacterial cell density of 10^6 CFU/ml (conversion factor, 5 ×

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TABLE 2 Antibiotic susceptibility of 30 *Y. pestis* strains

Antibiotic	Broth dilution MIC data at: 35°C Range	35°C MIC_{50}	35°C MIC_{90}	28°C Range	28°C MIC_{50}	28°C MIC_{90}	% agreement	Breakpoint % agreement (35°/28°)
Amikacin	0.25–4	1	2	0.25–8	2	8	25	12.5/50[a]
Gentamicin	0.06–1	0.5	1	0.06–2	1	2	50	25/50[b]
Netilmicin	0.12–2	0.5	1	0.06–4	0.5	1	100	12.5/12.5[a]
Streptomycin	1–8	2	4	1–16	4	8	50	100/50[b]
Tobramycin	0.06–1	0.5	1	0.06–2	0.5	1	100	25/25[a]
Azithromycin	1–16	4	8	1–64	16	32	25	
Cethromycin	0.25–4	1	2	1–16	4	8	25	
Telithromycin	0.5–8	2	4	0.5–32	4	16	25	
Clarithromycin	16–>64	64	64	4–>64	64	>64	100	
Garenoxacin	0.015–0.06	0.06	0.06	0.004–0.6	0.015	0.03	50	
Ciprofloxacin	0.008–0.12	0.015	0.03	0.004–0.03	0.008	0.015	50	30/1.5[b]
Gatifloxacin	0.015–0.12	0.06	0.06	0.004–0.06	0.03	0.03	50	30/1.5[a]
Gemifloxacin	0.004–0.03	0.015	0.03	0.002–0.03	0.008	0.03	100	12/12[a]
Levofloxacin	0.008–0.12	0.03	0.06	0.008–0.06	0.03	0.03	50	6/3[b]
Moxifloxacin	0.015–0.06	0.03	0.06	0.008–0.06	0.03	0.03	50	
Nalidixic acid	0.25–4	2	4	0.5–2	1	2	50	25/12.5[a]
Ofloxacin	0.015–0.25	0.06	0.12	0.008–0.12	0.03	0.06	50	6/3[a]
Sparfloxacin	0.004–0.06	0.015	0.03	0.002–0.03	0.008	0.015	50	
Novobiocin	8–>64	64	>64	8–>64	64	>64	100	
Amoxicillin-clavulanate (2:1)	0.03–1	0.25	0.5	0.06–0.5	0.25	0.25	50	6/6[a]
Amoxicillin	0.03–1	0.25	0.5	0.06–1	0.25	0.5	100	
Ampicillin	0.03–4	0.25	0.5	0.06–0.5	0.25	0.5	100	6/6[a]
Penicillin G	0.012–1	0.25	1	0.03–2	0.5	2	50	
Piperacillin	0.03–0.5	0.06	0.25	0.03–0.5	0.12	0.5	50	2/3[a]
Imipenem	0.12–1	0.25	0.5	0.06–1	0.25	0.5	100	
Ertapenem (Invanz)	0.004–0.06	0.03	0.03	0.002–0.03	0.015	0.03	100	
Faropenem	0.06–2	0.25	0.5	0.25–1	0.5	1	50	
Meropenem	0.015–0.12	0.06	0.12	0.015–0.06	0.06	0.06	50	
Cefepime	0.008–0.06	0.03	0.06	0.03–0.25	0.03	0.25	25	1/3[a]
Ceftazidime	0.03–0.5	0.12	0.25	0.03–0.25	0.06	0.12	50	3/1.5[a]
Cefotaxime	0.004–0.03	0.015	0.03	0.004–0.015	0.008	0.015	50	0.4/19[a]
Cefotetan	0.03–0.5	0.25	0.5	0.03–0.25	0.12	0.25	50	3/1.5[a]
Cefuroxime	0.03–2	0.5	2	0.06–2	0.5	1	50	25/12.5[a]
Cefazolin	0.25–8	2	4	1–4	2	4	50	8/25[a]
Ceftriaxone	0.008–0.03	0.015	0.03	0.008–0.03	0.015	0.015	50	0.4/19[a]
Aztreonam	0.008–0.06	0.03	0.03	0.008–0.03	0.015	0.03	100	0.4/38[a]
Sulfamethoxazole	0.5–>64	16	64	2−>64	16	>64	100	25/25[a]
Sulfamethoxazole-trimethoprim (19:1)	0.25–8	0.5	8	0.12–4	1	4	50	21/11[b]
Trimethoprim	0.12–16	0.5	8	0.12–8	0.5	2	25	1/25[a]
Doxycycline	0.06–2	0.5	1	0.06–0.5	0.25	0.5	50	25/12.5[b]
Tetracycline	0.25–2	0.5	2	1–16	4	8	25	50/50[b]
Tigecycline	0.06–0.5	0.25	0.25	0.03–0.5	0.12	0.5	50	
Rifampin	0.25–4	2	4	1–64	2	16	25	
Chloramphenicol	0.25–4	1	4	0.5–16	4	8	50	50/100[b]
Fusidic acid	16–>64	64	>64	2–>64	32	64	100	

[a] *Enterobacteriaceae* breakpoints were taken from reference 15.
[b] *Y. pestis* breakpoints were taken from reference 13.

10^8 CFU/ml/unit of optical density at 600 nm [OD_{600}]). To each well of the 96-well plate, 50 μl of this dilution was added for a final inoculum density of approximately 5×10^4 CFU/well (5×10^5 CFU/ml). The plates were incubated at either 28°C or 35°C and visually read at 24 and 48 h. Each MIC was determined in triplicate. Quality control of the antibiotic stocks was verified according to the CLSI methods by using *Staphylococcus aureus* strain ATCC 29213, *Pseudomonas aeruginosa* strain ATCC 27853, and *Escherichia coli* strain ATCC 25922 (9). All bacterial work was carried out under biosafety level 3 (BSL3) laboratory conditions.

RESULTS

Broth dilution MIC data are presented in Table 2. Because of the instability of the virulence plasmids at higher incubation temperatures (11, 12), determinations at 28°C were also performed. The majority of the MICs were within one well at the two temperatures, with only 17.77% (8/45) of the MIC_{90}s having a two-well difference. Agreements between the two temperatures ranged from 25% to 100%. MIC_{90} agreements with breakpoints varied

more widely, especially compared to the *Enterobacteriaceae* breakpoints. In general, the *Y. pestis* strains grew better at 28°C than at 35°C. This was expected since the low calcium response associated with the *Yersinia* virulence plasmid inhibits growth at the higher temperature.

DISCUSSION

The establishment of MICs for a number of defined and archived strains of *Y. pestis* will be helpful in serving as references in future testing. Few susceptibility breakpoints have been established for *Y. pestis*. The CLSI has developed some interpretive criteria based in part on these data, other published *in vitro* distribution data, and animal efficacy data (13). Standard testing at 35°C provided MIC_{90}s that indicate susceptibility to gentamicin, streptomycin, doxycycline, tetracycline, ciprofloxacin, levofloxacin, co-trimoxazole, and chloramphenicol based on those breakpoints (13), and these values agree with reference development data and data from a recent 392-strain study (10, 14). If the available *Enterobacteriaceae* breakpoints are used (15), most of the MIC_{90}s for the antibiotics evaluated would be considered susceptible. There are no breakpoints for the macrolides, novobiocin, or fusidic acid. However, the MIC_{90} values suggest that none of these antibiotics would be in a susceptible range, with the possible exception of cethromycin (MIC_{90}, 2 μg/ml). In a rat model, cethromycin showed some efficacy but only at very high doses that were well above the proposed human dose of 300 mg/day (16). Similar results have been observed in the mouse pneumonic plague model (H. S. Heine, unpublished data). These results suggest that an MIC of 1 to 2 μg/ml is higher than the efficacious breakpoint for *Y. pestis*. While the β-lactam and cephalosporin antibiotics demonstrate very good *in vitro* activity, animal efficacy data indicate that use of these antibiotics should be contraindicated (17). The equivalent results observed at 28°C may be significant. It was previously shown that the virulence plasmids in *Y. pestis* are unstable when grown at temperatures ranging from 35°C to 37°C (11, 12), which is the CLSI standard incubation temperature. While antibiotic resistance has not yet been associated with the virulence plasmids, the recent isolation of several *Y. pestis* strains containing multiple antibiotic resistance genes with transposable sequences (18, 19) raises the possibility of gene transfer to the virulence plasmids. To adequately assess the susceptibility profile of such a recombinant strain, to maintain the plasmid stability, and to obtain a reliable antibiotic reading, incubation at 28°C might be warranted. The observation in this study that susceptibility values did not shift significantly should be useful for reference laboratories.

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