JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2005, p. 5804–5807 Vol. 43, No. 11
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.11.5804–5807.2005

Broth Microdilution Susceptibility Testing of *Brucella* Species: Quality Control Limits for Ten Antimicrobial Agents against Three Standard Quality Control Strains

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Received 7 March 2005/Returned for modification 10 May 2005/Accepted 14 June 2005

Brucella broth without supplementation is the recommended medium for broth microdilution susceptibility tests of *Brucella abortus*, *B. melitensis*, and *B. suis*. Based on an eight-laboratory collaborative study using a pH-adjusted modification of this medium, we propose MIC quality control ranges for three control strains against 10 antimicrobials that are potentially efficacious for treating infections caused by these agents of bioterrorism.

Brucellosis is a disease primarily seen in domesticated animals, although human disease is still occasionally seen in certain Mediterranean areas and in a variety of third-world countries (10). Recently, *Brucella* spp. have been identified as belonging to a group of microorganisms that could be used as biological weapons of terrorism (16). The low incidence of naturally occurring brucellosis cases coupled with the hazardous nature of the organism has precluded the development of standardized susceptibility tests for *Brucella* spp. and has led to recommendations that *Brucella* spp. not be tested in the routine microbiology laboratory (8). The media have primarily included supplemented Mueller-Hinton agar (3, 17) or broth (7), Iso-Sensitest agar (9, 11), brucella broth and agar (5), or Trypticase soy broth (13). The majority of these studies were flawed in the respect that quality control (QC) ranges for these media do not exist, and therefore, quality control strains were rarely tested. Standardization of susceptibility testing methods for potential agents of bioterrorism such as *Brucella abortus*, *B. melitensis*, and *B. suis* is necessary for therapeutic guidance in the event of an outbreak with a potentially resistant isolate

TABLE 1. MIC ranges of *S. aureus* ATCC 29213 at 24 and 48 h of incubation

Antimicrobial agent	Incubation time (h)	No. of occurrences at indicated MIC (μg/ml)[a]																% of occurrences in range[b]
		<0.004	0.008	0.016	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	>32	64	128	256	
Gentamicin	24						0	0	36	64	46	53	37	4					NR[c]
	48						0	0	32	59	51	50	41	7					NR
Chloramphenicol	24								0	0	0	**4**	**210**	**26**	0				100
	48								0	0	0	**1**	**164**	**75**	0				100
Ciprofloxacin	24				0	0	0	**79**	**149**	**3**	9	0	0						96.3
	48				0	0	0	**4**	**198**	**17**	12	8	1						90.8
Doxycycline	24				0	10	**74**	**136**	**20**	0	0	0	0	0					95.8
	48				0	1	**19**	**136**	**83**	1	0	0	0	0					99.2
Levofloxacin	24				0	**3**	**114**	**111**	**12**	0	0	0	0						100
	48				0	0	**11**	**205**	**11**	13	0	0	0						94.6
Rifampin	24	237	1	2	0	0	0	0	0	0	0	0	0	0					NR
	48	212	24	4	0	0	0	0	0	0	0	0	0	0					NR
Azithromycin	24						0	0	0	0	**9**	**217**	**13**	1					99.6
	48						0	0	0	0	**4**	**132**	**100**	3					100
Tetracycline	24				0	0	**10**	**108**	**119**	**3**	0	0	0	0					100
	48				0	0	0	**26**	**175**	**39**	0	0	0	0					100
Streptomycin	24								0	0	0	4	**76**	**156**	**4**	0	0	0	98.3
	48								0	0	0	1	**57**	**160**	**21**	1	0	0	99.6
Trim/Sulfa[d]	24					0	0	6	18	16	3	12	10	7	168				NR
	48					0	0	0	0	0	0	1	1	9	208				NR

[a] The absence of a value represents the limit of MIC dilutions tested. Recommended QC ranges are represented in boldface characters.
[b] Percentage of results which fall within the recommended range (acceptable limit, ≥95%).
[c] NR, no range recommended due to extreme variability or off-scale mode.
[d] Trim/Sulfa, trimethoprim-sulfamethoxazole.

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TABLE 2. MIC ranges of *E. coli* ATCC 25922 at 24 and 48 h of incubation

Antimicrobial agent	Incubation time (h)	No. of occurrences at indicated MIC (μg/ml)[a]																	% of occurrences in range[b]
		<0.004	0.008	0.016	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	>16	32	64	128	256	
Gentamicin	24						0	0	0	**4**	**112**	**60**	**58**	6					97.5
	48						0	0	0	**2**	**103**	**65**	**56**	14					94.2
Chloramphenicol	24								0	0	**2**	**229**	**24**	0	0				100
	48								0	0	1	**84**	**154**	**0**	0				99.6
Ciprofloxacin	24				235	3	2												NR[c]
	48				235	3	2												NR
Doxycycline	24				0	0	0	0	**1**	**237**	**17**	0	0						100
	48				0	0	0	0	1	**49**	**190**	**0**	0						99.6
Levofloxacin	24				236	3	1	0	0	0	0	0	0						NR
	48				233	4	3	0	0	0	0	0	0						NR
Rifampin	24	0	0	0	0	0	0	0	0	0	9	**25**	**206**	**0**					96.3
	48	0	0	0	0	0	0	0	0	0	9	**15**	**211**	**5**					96.3
Azithromycin	24						0	0	0	0	4	8	107	121					NR
	48						0	0	0	0	3	8	85	144					NR
Tetracycline	24				0	0	0	0	**30**	**176**	**34**	0	0	0					100
	48				0	0	0	0	**0**	**109**	**94**	**37**	0	0					100
Streptomycin	24								0	0	0	**0**	**112**	**126**	**2**	0	0	0	100
	48								0	0	0	**0**	**97**	**137**	**6**	0	0	0	100
Trim/Sulfa[d]	24					0	0	22	42	12	23	68	38	11	8				NR
	48					0	0	16	31	12	8	35	86	24	12				NR

[a] The absence of a value represents the limit of MIC dilutions tested. Recommended QC ranges are represented in boldface characters.
[b] Percentage of results which fall within the recommended range (acceptable limit, ≥95%).
[c] NR, no range recommended due to extreme variability or off-scale mode.
[d] Trim/Sulfa, trimethoprim-sulfamethoxazole.

as well as to provide validation to investigations into the in vitro activity of newer compounds. The purpose of this study was to propose quality control ranges for 10 antimicrobial agents diluted in pH-adjusted brucella broth without additional supplementation against three QC strains recommended by the Clinical Laboratory Standards Institute (CLSI; formerly the NCCLS) for use in incubation conditions conducive to the growth of *Brucella*.

MIC testing was performed according to the recommendations of the CLSI (14, 15). Broth microdilution trays were commercially prepared by TREK Diagnostic Systems (Westlake, OH) to contain serial dilutions of multiple drugs diluted in each of three different lots of unsupplemented brucella broth. The pH of the broth was adjusted to a range of 7.1 ± 0.1. This pH range was selected so that the activity of certain pH-sensitive antimicrobials, such as gentamicin, azithromycin,

TABLE 3. MIC ranges of *S. pneumoniae* ATCC 49619 at 24 and 48 h of incubation

Antimicrobial agent	Incubation time (h)	No. of occurrences at indicated MIC (μg/ml)[a]																	% of occurrences in range[b]
		<0.004	0.008	0.016	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	>16	32	64	128	256	
Gentamicin	24						0	0	0	0	0	0	12	228					NR[c]
	48						0	0	0	0	0	0	6	234					NR
Chloramphenicol	24								0	**2**	**108**	**127**	**3**	0	0				100
	48				0	0	0	0	0	0	**65**	**167**	**8**	0	0				100
Ciprofloxacin	24				0	0	0	**20**	**164**	**56**	0	0	0						100
	48							**4**	**106**	**126**	**4**	0	0						100
Doxycycline	24				**62**	**113**	**59**	6	0	0	0	0	0	0					97.5
	48				**6**	**97**	**126**	**11**	0	0	0	0	0	0					97.5
Levofloxacin	24				0	0	0	**1**	**153**	**86**	0	0	0						100
	48				0	0	0	**0**	**100**	**140**	**0**	0	0						100
Rifampin	24	2	**57**	**115**	**60**	4	1	0	0	0	0	0	0	0					97.1
	48	1	**19**	**63**	**131**	**24**	1	0	0	0	0	0	0	0					99.2
Azithromycin	24						**7**	**67**	**164**	**0**	1	1	0	0					96.7
	48						**4**	**30**	**197**	**7**	2	0	0	0					97.5
Tetracycline	24				**0**	**74**	**106**	**57**	2	0	0	0	0	0					98.8
	48				0	**38**	**106**	**94**	**2**	0	0	0	0	0					100
Streptomycin	24								0	0	0	0	2	**32**	**128**	**70**	4	0	97.5
	48								0	0	0	0	1	**19**	**96**	**113**	**9**	0	99.6
Trim/Sulfa[d]	24					0	0	0	**37**	**174**	**29**	0	0	0	0				100
	48					0	0	0	**18**	**176**	**39**	7	0	0	0				100

[a] The absence of a value represents the limit of MIC dilutions tested. Recommended QC ranges are represented in boldface characters.
[b] Percentage of results which fall within the recommended range (acceptable limit, ≥95%).
[c] NR, no range recommended due to extreme variability or off-scale mode.
[d] Trim/Sulfa, trimethoprim-sulfamethoxazole.

TABLE 4. Recommended QC MIC ranges for *S. aureus*, *E. coli*, and *S. pneumoniae* using pH-adjusted brucella broth without supplements

Antimicrobial agent	MIC range (μg/ml) at indicated incubation time (h)					
	S. aureus ATCC 29213		*E. coli* ATCC 25922		*S. pneumoniae* ATCC 49619	
	24	48	24	48	24	48
Gentamicin	NR[a]	NR	1–8	1–8	NR	NR
Chloramphenicol	4–16	4–16	2–8	4–16	1–8	2–8
Ciprofloxacin	0.25–1	0.25–1	NR	NR	0.25–1	0.25–2
Doxycycline	0.12–0.5	0.12–0.5	0.5–2	1–4	0.03–0.12	0.03–0.25
Levofloxacin	0.06–0.5	0.12–0.5	NR	NR	0.25–1	0.25–2
Rifampin	NR	NR	4–16	4–16	0.008–0.03	0.008–0.06
Azithromycin	2–8	2–16	NR	NR	0.25–1	0.25–1
Tetracycline	0.12–1	0.25–1	0.5–2	0.5–4	0.03–0.25	0.06–0.5
Streptomycin	8–64	8–64	4–32	4–32	16–64	16–128
Trimeth/Sulfa[b]	NR	NR	NR	NR	0.5–2	0.5–2

[a] NR, no range recommended due to extreme variability or off-scale mode.
[b] Trimeth/Sulfa, trimethoprim-sulfamethoxazole.

and streptomycin, would not be severely compromised. The antimicrobial agents are listed in Tables 1 to 3. The trays were then frozen and shipped to the eight participating laboratory sites which are identified in Acknowledgments. On separate days of testing, each of the three QC strains, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Streptococcus pneumoniae* ATCC 49619, was inoculated into the MIC trays. The MIC trays were incubated at 35°C in ambient air and read visually at 24 h and again at 48 h. Each study site tested the three organisms in three separate lots of brucella broth for 10 consecutive days. During the study, laboratories performed colony counts to insure proper inoculation concentrations. The median colony counts were 5.0×10^5 (range, 1.7 to 10.0) for *S. aureus* ATCC 29213; 3.8×10^5 (range, 0.6 to 8.3) for *E. coli* ATCC 25922; and 3.8×10^5 (range, 0.6 to 6.3) for *S. pneumoniae* ATCC 49619.

Tables 1 to 3 represent the frequency distributions for the MICs of the antimicrobial agents tested with each of the three quality control strains. Significant lot-to-lot variability was observed among results obtained for gentamicin and trimethoprim-sulfamethoxazole against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 only. MICs read at 24 and 48 h differed by no more than 1 $\log_2$ dilution interval. A three-dilution range was proposed whenever there was a unimodal distribution of the values, and a four-dilution range was proposed whenever there was a bimodal distribution of results (2, 15). No ranges were proposed for antimicrobials which had modal MICs which were off the scale at either extreme. With three exceptions, the MICs for all strains attained ≥95% distribution within the proposed ranges. The exceptions were gentamicin versus *E. coli* ATCC 25922 at 48 h (94.2% included), levofloxacin versus *S. aureus* ATCC 29213 at 48 h (94.6% included), and ciprofloxacin versus *S. aureus* ATCC 29213 at 48 h (90.8% included). All of the ciprofloxacin versus *S. aureus* ATCC 29213 values that were off the scale were from a single laboratory. If these results were to be excluded from the analysis, then 100% of the remaining values would be within the proposed ranges. No ranges were proposed for trimethoprim-sulfamethoxazole versus *E. coli* ATCC 25922 or *S. aureus* ATCC 29213 because of the excessive lot-to-lot variability of the results. The results for *S. pneumoniae* ATCC 49619 were less variable, and a three-dilution range could be successfully proposed.

Based on the data provided by the eight laboratories, the Subcommittee on Antimicrobial Susceptibility Testing of the CLSI approved the QC ranges listed in Table 4. MIC results for *Brucella* spp. read after 24 h of incubation should use the 24-h QC ranges; results read after 48 h should use only the 48-h QC ranges.

The choice of antimicrobial agents in this study focused on the reported activities of these agents in the treatment of brucellosis in humans. Although doxycycline plus streptomycin or rifampin is the preferred therapeutic agent (1, 4, 7, 12), newer antimicrobial agents with greater bactericidal and intracellular activity, including newer fluoroquinolones (6, 7), are now being considered. The in vitro effectiveness of these newer agents has yet to be confirmed by human clinical trials.

We are grateful to the following participating individuals and laboratories: S. Jenkins, Carolinas Medical Center, Charlotte, NC; D. Hardy and M. Tolomay, University of Rochester Medical Center, Rochester, NY; J. Hindler, UCLA, Los Angeles, CA; C. Knapp and S. Killian, TREK Diagnostic Systems, Cleveland, OH; G. Procop and M. Tuohy, Cleveland Clinic, Cleveland, OH; R. Rennie and C. Brosnikoff, University of Alberta Hospital, Alberta, Canada; and F. Tenover, J. Swenson, and J. Patel, CDC, Atlanta, GA.

This study was supported by a grant from PhRma, Inc., Washington, D.C.

REFERENCES

1. **Ariza, J., F. Gudiol, R. Pallares, P. F. Viladrich, G. Rufi, J. Corredoira, and M. R. Miravitlles.** 1992. Treatment of human brucellosis with doxycycline plus rifampin or doxycycline plus streptomycin. A randomized, double-blind study. Ann. Intern. Med. **117:**25–30.
2. **Barry, A. L., P. C. Fuchs, R. N. Jones, et al.** 1989. Statistical criteria for selecting quality control limits for broth microdilution susceptibility tests with 39 different antimicrobial agents. Diagn. Microbiol. Infect. Dis. **12:**413–420.
3. **Garcia-Rodriguez, J. A., J. L. Munoz Bellido, M. J. Fresnadillo, and I. Trujillano.** 1993. In vitro activities of new macrolides and rifapentine against *Brucella* spp. Antimicrob. Agents Chemother. **37:**911–913.
4. **Hall, W. H.** 1990. Modern chemotherapy for brucellosis in humans. Rev. Infect. Dis. **12:**1060–1099.
5. **Hall, W. H., and R. E. Manion.** 1970. In vitro susceptibility of *Brucella* to various antibiotics. Applied Microbiol. **20:**600–604.
6. **Hussain, Q. S. M., M. Akhtar, Y. Ueno, and M. B. Al-Sibai.** 1989. Susceptibility of *Brucella melitensis* to fluoroquinolones. Drugs Exp. Clin. Res. **15**(10)**:**483–485.
7. **Khan, M. Y., M. Dizon, and F. W. Kiel.** 1989. Comparative in vitro activities of ofloxacin, difloxacin, ciprofloxacin, and other selected antimicrobial agents against *Brucella melitensis*. Antimicrob. Agents Chemother. **33:**1409–1410.
8. **King, A.** 2001. Recommendations for susceptibility tests on fastidious organisms and those requiring special handling. J. Antimicrob. Chemother. **48**(Suppl. S1)**:** 77–80.
9. **Landinez, R., J. Linares, E. Loza, J. Martinez-Beltran, R. Martin, and F. Baquero.** 1992. In vitro activity of azithromycin and tetracycline against 358 clinical isolates of *Brucella melitensis*. Eur. J. Clin. Microbiol. Infect. Dis. **11:**265–267.
10. **Lang, R., M. Banai, M. Lishner, and E. Rubinstein.** 1995. Brucellosis. Int. J. Antimicrob. Agents **5:**203–208.
11. **Lopez-Merino, A., A. Contreras-Rodriguez, R. Migranas-Ortiz, R. Orrantia-Gradin, G. M. Hernandez-Oliva, A. T. Y. Gutierrez-Rubio, and O. Cardenosa.** 2004. Susceptibility of Mexican Brucella isolates to moxifloxacin, ciprofloxacin and other antimicrobials used in the treatment of human brucellosis. Scand. J. Infect. Dis. **36:**636–638.
12. **Montejo, J. M., I. Alberola, P. Glez-Zarate, A. Alvarez, J. Alonso, A. Canovas, and C. Aguirre.** 1993. Open, randomized therapeutic trial of six antimicrobial regimens in the treatment of human brucellosis. Clin. Infect. Dis. **16:**671–676.
13. **Mortensen, J. E., D. G. Moore, J. E. Clarridge, and E. J. Young.** 1986. Antimicrobial susceptibility of clinical isolates of *Brucella*. Diagn. Microbiol. Infect. Dis. **5:**163–169.
14. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aer-

obically. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
15. **National Committee for Clinical Laboratory Standards.** 2001. Development of in vitro susceptibility testing criteria and quality control parameters. Approved guideline M23-A2. National Committee for Clinical Laboratory Standards, Wayne, Pa.
16. **Robinson-Dunn, B.** 2002. The microbiology laboratory's role in response to bioterrorism. Arch. Pathol. Lab. Med. **126:**291–294.
17. **Trujillano-Martin, I., E. Garcia-Sanchez, I. M. Martinez, M. J. Fresnadillo, J. E. Garcia-Sanchez, and J. A. Garcia-Rodriguez.** 1999. In vitro activities of six new fluoroquinolones against *Brucella melitensis*. Antimicrob. Agents Chemother. **43:**194–195.