NOTES

Comparison of Etest Method with Reference Broth Microdilution Method for Antimicrobial Susceptibility Testing of *Yersinia pestis*[▽]

David R. Lonsway,^{1*} Sandra K. Urich,² Henry S. Heine,^{3†} Sigrid K. McAllister,¹
Shailen N. Banerjee,¹ Martin E. Schriefer,² and Jean B. Patel¹

Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Atlanta, Georgia 30333¹; Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Ft. Collins, Colorado 80521²; and United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Ft. Detrick, Maryland 21702³

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The utility of Etest for antimicrobial susceptibility testing of *Yersinia pestis* was evaluated in comparison with broth microdilution and disk diffusion for eight agents. Four laboratories tested 26 diverse strains and found Etest to be reliable for testing antimicrobial agents used to treat *Y. pestis*, except for chloramphenicol and trimethoprim-sulfamethoxazole. Disk diffusion testing is not recommended.

Yersinia pestis is the etiologic agent of the plague and has potential for use as a biological weapon (1, 13, 17–20, 23). Because of this, it is important that emerging drug resistance, whether natural or engineered, should be detectable using standardized methods that are easily implemented in multiple laboratories. The Clinical and Laboratory Standards Institute (CLSI) describes a reference broth microdilution (BMD) method for antimicrobial susceptibility testing of *Y. pestis* and provides MIC interpretive guidelines for eight antimicrobial agents (6). BMD uses cation-adjusted Mueller-Hinton broth (CAMHB) and requires incubation at 35°C for 24 h with an option for incubation for 48 h when growth at 24 h is insufficient for endpoint interpretation. Unfortunately, reference BMD is difficult to incorporate in many laboratories because it is relatively costly and laborious and requires the storage of panels in a frozen or dehydrated format. Several alternative susceptibility testing methods exist, including the disk diffusion and Etest methods. However, before these methods can be employed for a species, they must be evaluated and compared to BMD to determine correlations between the results obtained by comparisons of the methods.

Y. pestis isolates are fastidious and may grow more slowly on artificial media than other common species of *Enterobacteriaceae*, and so susceptibility testing methods for *Y. pestis* have been difficult to standardize. Several methods are described in the literature. Disk diffusion testing has generally been performed on Mueller-Hinton agar (MHA), typically using 48 h of incubation at 35°C, but methodological descriptions are sometimes lacking in detail (10, 16, 24). The Etest method was employed by Wong et al. (25) using MHA with 5% sheep

blood, incubation at 35°C, and an inoculum matching a no. 1 McFarland instead of the 0.5 McFarland standard used in most disk or Etest diffusion studies. Agar dilution, using MHA incubated at 27° to 30°C for 48 h, is the most common method reported in the literature (7, 8, 11, 12, 22). Broth macrodilution and microdilution methods have also been used with various incubation temperatures (2, 21).

There are several attributes of disk diffusion and Etest methods that make them attractive alternative methods for susceptibility testing, including ease of storage and a long shelf life for the disks and strips. Also, these are agar-based methods and the endpoints can be easier to read than those of BMD. The Etest has the added benefit of producing an MIC result. In this report, we present results of a multicenter study comparing Etest and disk diffusion methods with the CLSI reference BMD method for susceptibility testing of *Y. pestis*.

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Twenty-six diverse *Y. pestis* strains from the Centers for Disease Control and Prevention (CDC) and U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) collections were tested by both the Etest and BMD methods at four test sites and additionally by disk diffusion at two of these sites. Six strains were biovar Antiqua, seven were biovar Medievalis, and 12 were biovar Orientalis; one atypical isolate could not be assigned to any biovar. The antimicrobial agents were tested by both BMD and Etest (bioMérieux, Durham, NC), and their corresponding ranges were as follows for BMD and Etest, respectively: for chloramphenicol, 0.03 to 64 µg/ml and 0.016 to 256 µg/ml; for ciprofloxacin, 0.03 to 64 µg/ml and 0.002 to 32 µg/ml; for doxycycline, 0.03 to 64 µg/ml and 0.016 to 256 µg/ml; for gentamicin, 0.03 to 64 µg/ml and 0.016 to 256 µg/ml; for levofloxacin, 0.06 to 64 µg/ml and 0.002 to 32 µg/ml; for streptomycin, 0.03 to 64 µg/ml and 0.016 to 256 µg/ml; for tetracycline, 0.03 to 64 µg/ml and 0.016 to 256 µg/ml; and for trimethoprim-sulfamethoxazole, 0.015/32 to 16/304 µg/ml and

* Corresponding author. Mailing address: Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention, Mailstop G08, 1600 Clifton Rd., Atlanta, GA 30333. Phone: (404) 639-2825. Fax: (404) 639-1381. E-mail: dul7@cdc.gov.

† Present address: Ordway Research Institute Inc., Albany, NY.

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TABLE 1. MIC₉₀, MIC range, and interpretive category agreement for 48-h Etest and 48-h broth microdilution MICs for eight antimicrobial agents tested against 26 *Y. pestis* isolates at four test sites (104 test results)

Antimicrobial agent	BMD MIC (μg/ml)		Etest ^a MIC (μg/ml)		MIC breakpoint ^b			% category agreement (% minor errors)
	MIC ₉₀	Range	MIC ₉₀	Range	S	I	R	
Chloramphenicol	8	0.25–16	2	0.12–4	≤8	16	≥32	98 (2)
Ciprofloxacin	0.12	≤0.03–0.5	0.06	0.008–0.12	≤0.25			99 (1) ^c
Doxycycline	2	0.12–4	2	0.25–2	≤4	8	≥16	100
Gentamicin	1	0.06–4	1	0.12–1	≤4	8	≥16	100
Levofloxacin	≤0.06	≤0.06–0.12	0.06	0.008–0.12	≤0.25			100
Streptomycin	4	1–8	4	1–8	≤4	8	≥16	97 (3)
Tetracycline	2	0.25–4	2	0.25–4	≤4	8	≥16	100
Trimethoprim-sulfamethoxazole ^d	0.06	≤0.015–0.25	0.03	0.008–0.06	≤2		≥4	100

^a Etest MICs rounded up to correspond to log₂ broth microdilution (BMD) series.^b S, susceptible; I, intermediate; R, resistant.^c One Etest MIC = 0.06 μg/ml (susceptible); BMD MIC = 0.5 μg/ml (nonsusceptible).^d Only the trimethoprim portion of the 1:19 combination is displayed.

0.002/0.038 to 32/608 μg/ml. Because the ranges for the BMD testing of some drugs did not encompass the lower concentrations obtainable by the Etest, essential agreement (± 1 log₂ dilution) between methods was considered to be off the scale for some result comparisons (e.g., a BMD levofloxacin MIC of ≤0.06 μg/ml and an Etest MIC of 0.03 μg/ml). At CDC, 96-well MIC trays were prepared using 100 μl of CAMHB (BBL, Sparks, MD) per well; trays were kept frozen at –70°C and shipped to participating laboratories. Testing was performed by CLSI standard methods for BMD and disk diffusion (4–6). Inocula were prepared from 18- to 24-h aerobic cultures grown on 5% sheep blood agar plates (BBL) by the direct colony suspension method in Mueller-Hinton broth (MHB) (Remel, Lenexa, KS) to equal a 0.5 McFarland turbidity standard (4); the same inocula were used to inoculate 150-mm-diameter MHA plates for Etest and disk diffusion tests. Immediately after inoculation, at least two random colony counts were performed at each test site with the positive BMD growth control well in order to assess inoculum size. No more than four Etest strips were applied to a plate. For disk diffusion, commercial disks (BBL) were applied with a self-tamping multidisk dispenser (BBL). BMD panels and MHA plates for both Etest and disk diffusion were incubated at 35°C and read at 24- and 48-h time periods. Etests were read as directed according to the Etest package inserts as follows: chloramphenicol, doxycycline, tetracycline, and trimethoprim-sulfamethoxazole were read at 80% inhibition for the intersection point; ciprofloxacin, levofloxacin, gentamicin, and streptomycin were read at 100% inhibition. For data analyses, Etest MICs were rounded up to the nearest log₂ dilution. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used for BMD and Etest quality control. MICs for quality control strains were determined by incubation for 16 to 20 h. Acceptable BMD quality control ranges for streptomycin were previously established at the CDC (unpublished data). Quality control of the chloramphenicol Etest was performed by testing *Escherichia coli* ATCC 25922 and applying the acceptable range for the CLSI BMD.

To measure agreement between the Etest and BMD results, the distribution of differences in the log₂ dilution MICs was examined and the percentage of MIC determinations that yielded identical values (essential agreement within the accuracy limits of the reference method [± 1 log₂ dilution]) was

calculated for each drug. Also, to determine whether the Etest method produced significantly lower or higher MICs than the reference method, we performed a Wilcoxon signed-rank test with the log₂ dilution MICs of the two tests by the use of SAS statistical software (SAS Institute Inc., Cary, NC); MICs within ± 1 log₂ dilution were regarded as identical for this test. Comparison of interpretative category results (susceptible, intermediate, and resistant) was done by calculating rates of minor, major, and very major errors.

In general, MIC endpoints were more easily discernible by BMD than by Etest at 24 h (data not shown). At three test sites (A, B, and C), nearly all BMD and Etest MICs were readable at 24 h of incubation; a single Etest result was the exception. Site D reported readable BMD MICs at 24 h for all but one strain. There was insufficient growth to read 24-h Etest MICs for eight strains at site D. The numbers of Etest MICs that were unreadable due to inadequate growth at 24 h by drug were as follows: for chloramphenicol, $n = 6$; for ciprofloxacin, $n = 4$; for doxycycline, $n = 3$; for gentamicin, $n = 2$; for levofloxacin, $n = 5$; for streptomycin, $n = 5$; for tetracycline, $n = 4$; and for trimethoprim-sulfamethoxazole, $n = 4$. All test sites were able to read all results at 48 h. Therefore, 48-h results were used in the comparison. Colony counts demonstrated that inoculum densities in the BMD wells were within acceptable limits for all four test sites; the averages for the sites ranged from 1.1×10^5 CFU/ml to 4.2×10^5 CFU/ml.

The MIC₉₀ and the MIC range for each antimicrobial agent were compared by method (Table 1). For all drugs except chloramphenicol, the MIC₉₀s for all of the methods were the same or within ± 1 log₂ dilution of one another. Category agreement between the Etest and BMD methods for all drugs was excellent at 97% to 100%; all errors were minor (Table 1). All BMD and Etest MICs were within the susceptible range after 24 h of incubation (data not shown). At 48 h, most MICs were within the susceptible range except for the following nonsusceptible results: chloramphenicol ($n = 2$); ciprofloxacin ($n = 1$); and streptomycin ($n = 5$). Most of the nonsusceptible results were from the BMD method rather than Etest (Fig. 1).

The two methods are best compared by analyzing essential agreement (the percentages of MICs within ± 1 log₂ dilution for the two methods [Table 2]). Essential agreement for all sites combined (including off-scale MICs) was $\geq 90\%$ for ciprofloxacin, doxycycline, levofloxacin, streptomycin, and tetra-

FIG. 1. Numbers of MIC results obtained by using 48-h Etest and 48-h broth microdilution (BMD) for eight antimicrobial agents tested against 26 *Y. pestis* isolates at four test sites ($n = 104$). The lowest dilution tested by BMD is indicated for cases in which the value was different from the lowest dilution on the x axis. Trimethoprim-sulfamethoxazole MIC values indicate the trimethoprim portion only.

TABLE 2. Comparison of 48-h Etest with 48-h broth microdilution MICs for eight antimicrobial agents tested against 26 *Y. pestis* isolates at four test sites (104 test results)

Antimicrobial agent	No. of results with indicated MIC log ₂ dilution differences between Etest and BMD reference method ^a						% essential agreement ^b (% including off-scale MICs)	<i>P</i> value ^d
	≤−3	−2	−1	0	+1	+2		
Chloramphenicol	25	43	27	8	1		35	<0.001
Ciprofloxacin ^c	4	6	39	53	2		87 (90)	<0.001
Doxycycline		1	14	48	35	6	93	0.03
Gentamicin	1	2	18	47	26	5	88	0.03
Levofloxacin ^c			6	98			100 (100)	N/A ^e
Streptomycin			25	72	7		100	N/A
Tetracycline		2	26	47	27	2	96	0.5
Trimethoprim-sulfamethoxazole ^c	3	20	61	20			78 (78)	<0.001

^a −1, Etest MIC is 1 log₂ dilution lower than broth microdilution (BMD) MIC; +1, Etest MIC is 1 log₂ dilution higher than BMD MIC; 0, no difference between Etest MIC and BMD MIC, etc. Etest MICs were rounded up to correspond to log₂ BMD dilution series.

^b Values represent percent essential agreement when the Etest MIC was within ± 1 log₂ dilution of the corresponding BMD MIC; on-scale MICs used for calculation. ^c Drug had off-scale Etest MICs less than or equal to the lowest tested corresponding BMD MIC. Ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazole had 27, 96, and 1 off-scale MICs, respectively; the other drugs had no off-scale results.

^d *P* values represent the probabilities obtained with Wilcoxon signed-rank test (a difference of ± 1 log₂ dilution is assumed to represent a difference of zero); differences were significant when *P* ≤ 0.05. (For example, 10 ciprofloxacin Etest MICs were more than 1 log₂ dilution lower than their corresponding BMD MICs, while none were more than 1 log₂ dilution higher than the BMD MIC; this difference was significant [*P* < 0.001].)

^e N/A, not applicable.

cycline. The values for essential agreement for gentamicin, trimethoprim-sulfamethoxazole, and chloramphenicol were lower, at 88%, 78%, and 35%, respectively. Except for chloramphenicol and trimethoprim-sulfamethoxazole, there was generally little or no variation in essential agreement between sites for each drug (Table 3). For chloramphenicol and trimethoprim-sulfamethoxazole, essential agreement between MIC methods at individual sites ranged from 4% to 81% and from 58% to 96%, respectively.

The gentamicin and doxycycline Etest MICs were significantly (*P* = 0.03) higher than the corresponding BMD MICs (Table 2); however, the values for essential agreement between methods for each drug were still good at 88% and 93%, respectively (Table 2). Chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole Etest MICs were, on average, significantly (*P* < 0.001) lower than their corresponding BMD MICs, and only ciprofloxacin had an essential agreement of ≥90%. For levofloxacin, streptomycin, and tetracycline, no statistical differences were seen between Etest and BMD MICs.

For disk diffusion, all antimicrobial disks except the strep-

tomycin disks produced large-diameter zones; some zones exceeded 40 to 50 mm in diameter (Table 4) and overlapped one another on the MHA plate. Zone margins for drugs producing these large zones were often fuzzy or indistinct. Two isolates at one test site had unreadable zones for all drugs due to poor growth at 24 h. Chloramphenicol and trimethoprim-sulfamethoxazole disks occasionally produced a double-zone effect with a lighter inner margin of growth, possibly due to the static activity of the drug with this slowly growing organism. Streptomycin disks produced the lowest (23- to 24-mm-diameter) average disk zone inhibition size and the most distinct, readable zones. Zone sizes of the other disk types averaged 30 to 42 mm in diameter at 48 h, and their diameters were more difficult to read.

In general, there was good essential agreement between the Etest method and the reference BMD method for antimicrobial susceptibility testing. However, essential agreement rates between Etest and BMD for chloramphenicol, gentamicin, and trimethoprim-sulfamethoxazole were lower than the 90% minimum that is recommended by the U.S. Food and Drug Administration

TABLE 3. Comparison of essential agreement at four test sites between 48-h Etest MICs and 48-h broth microdilution MICs for eight antimicrobial agents tested against 26 *Y. pestis* isolates

Test site(s)	% essential agreement ^a						
	CHL	CIP	DOX	GEN	LVX	STR	TET
A	4	85	100	81	100	100	85
B	4	92	96	77	100	100	73
C	81	100	85	96	100	100	92
D	50	85	92	96	100	100	92
All sites	35	90	93	88	100	100	96

^a Values represent percent essential agreement when the Etest MIC was within ±1 log₂ dilution of the reference broth microdilution MIC; data include off-scale results for CIP, LVX, and SXT. There were 26 MICs per site for each drug (total, *n* = 104 for all sites combined). CHL, chloramphenicol; CIP, ciprofloxacin; DOX, doxycycline; GEN, gentamicin; LVX, levofloxacin; STR, streptomycin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole.

TABLE 4. Disk diffusion results for tests performed using Mueller-Hinton agar and 26 *Y. pestis* isolates at two sites^a

Antimicrobial disk (concn [μg])	Inhibition zone diam (mm) for indicated incubation period (h)			
	Range		Avg	
	24	48	24	48
Chloramphenicol (30)	28–47	25–51	39	38
Ciprofloxacin (5)	30–51	30–53	42	42
Doxycycline (30)	26–40	26–41	33	33
Gentamicin (10)	24–36	22–42	29	30
Levofloxacin (5)	34–48	34–50	41	42
Streptomycin (10)	17–30	18–32	23	24
Tetracycline (30)	27–40	26–39	32	33
Trimethoprim-sulfamethoxazole (1.25–23.75)	34–50	34–49	43	42

^a Two of the 26 isolates had unreadable growth at 24 h at one site.

(FDA) for use of a new method in clinical testing (<http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm071462.pdf>).

Differences in lighting intensity in the biological safety cabinet at each test site may be a contributing factor for endpoint determination. We have found that reading an Etest endpoint is easier when a reading lamp is placed in the cabinet to supplement the light source. All sites except site D utilized this extra light source to enhance readability of the susceptibility tests. Tetracycline and doxycycline Etests, like those of chloramphenicol and trimethoprim-sulfamethoxazole, were also read at an 80% endpoint, but no double ellipses were noticeable and the essential agreement was $\geq 90\%$ for both tetracycline-class drugs, so it appears that this Etest phenomenon varies by drug class for *Y. pestis*.

For gentamicin, the overall essential agreement was 88%, just below the recommended 90% agreement cutoff. Two sites demonstrated high (96%) essential agreement, but the agreement was lower (77% and 81%) for the other two sites. The reason for this variability is unknown. However, overall MIC distributions for Etest and BMD MICs are similar (Fig. 1). It has been suggested that, for new susceptibility methods that are performed with slowly growing or fastidious organisms, testing should be less rigidly held to this standard and that a lower essential agreement is permissible (14). This logic appears appropriate for the gentamicin Etest, since the essential agreement was 88%. The acceptability of the trimethoprim-sulfamethoxazole Etest at 78% essential agreement is unclear.

A limitation of this study was that no isolates with known resistance were included. Access to the Madagascar drug-resistant strains (3, 9–11) is restricted. Other reports of drug resistance in *Y. pestis* are rare, and resistant strains are not well characterized (15, 16, 24). Therefore, we could not evaluate the ability of the Etest method to detect known resistance in *Y. pestis*.

The disk diffusion method is not recommended for *Y. pestis* because of the difficulty in reading the poorly defined zones of inhibition and large zone diameters encountered with most of the drugs tested. Streptomycin disk testing may warrant further study if streptomycin-resistant isolates become accessible, because the zone sizes were smaller and more distinct than those obtained with other disks.

In summary, in a comparison of two MIC methods for *Y. pestis* susceptibility testing, results for all antimicrobial agents correlated well between Etest and BMD except for chloramphenicol and trimethoprim-sulfamethoxazole, for which endpoints were difficult to determine by Etest. There was also greater site-to-site variability for chloramphenicol and trimethoprim-sulfamethoxazole than for the other drugs. The Etest method appears to be an acceptable alternative to BMD for ciprofloxacin, doxycycline, gentamicin, levofloxacin, streptomycin, and tetracycline but not for chloramphenicol and trimethoprim-sulfamethoxazole. Since *Y. pestis* grew slowly on MHA, 48 h of incubation is recommended for Etest. However, as we were unable to include any drug-resistant *Y. pestis* strains in our study, it is also recommended that nonsusceptible Etest MICs be confirmed by a reference BMD MIC test. We also advise that confirmatory BMD testing be performed on any *Y.*

pestis isolate with a ciprofloxacin or levofloxacin Etest MIC of 0.25 $\mu\text{g/ml}$, an MIC at the high end of the susceptible range, as it is unknown whether emerging resistance can be detected for these two drugs by the Etest method.

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