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Re-Evaluation of Clinical and Laboratory Standards Institute (CLSI) Disk Diffusion Breakpoints for Tetracycline and Doxycycline When Testing Enterobacteriaceae

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ABSTRACT

Background:

CLSI *Acinetobacter* spp. disk diffusion (DD) breakpoints (BPs) for tetracycline (TET) and doxycycline (DOX) were recently re-evaluated and modified due to high rates of discordant results compared to reference broth microdilution (BMD) methods. We extended this re-evaluation to Enterobacteriaceae (ENT) DD BPs for these tetracyclines, which currently (2006) are ≥19 and ≤14 mm for TET and ≥16 and ≤12 mm for DOX, for susceptible (S) and resistant (R) respectively.

Methods:

454 recent clinical isolates were studied, including *E. coli* (100), *K. pneumoniae* (100), *K. oxytoca* (KOX; 50), *Enterobacter* spp. (61), *Citrobacter* spp. (57), *P. mirabilis* (24), *Serratia* spp. (SM; 24), indole-positive *Proteae* (IPP; 28) and others (10). Isolates were tested against TET and DOX by DD and BMD methods according to CLSI guidelines. Regression plot analyses determined inter-method accuracy for current DD BPs.

Results:

S and R rates were 67.9 and 27.5% for TET and 68.9 and 23.1% for DOX, respectively. Regression plot analyses showed excellent (r) values of 0.95 for TET and 0.92 for DOX. DD error rates (minor/major/very major) were 15.0/0.9/0.0% for TET and 11.5/0.4/0.0% for DOX when using current CLSI BPs, and 4.6/0.0/0.0% for TET and 5.5/0.0/0.0% for DOX when BPs were adjusted to (S/R) ≥14/≤10 mm for TET and ≥13/≤9 mm for DOX. Using published CLSI BPs (2006), the highest rates of discrepant results were observed among SM (50- 58%) and IPP (21-32%) isolates; while highest agreements were observed for *E. coli* (98% for TET) and KOX (94% for DOX).

Conclusions:

The current CLSI DD BPs provide unacceptable rates of errors when testing TET and DOX against ENT. CLSI should consider the proposed changes in order to improve the accuracy of DD for testing tetracycline derivatives against ENT as previously accomplished for *Acinetobacter* testing.

INTRODUCTION

The tetracyclines were the initial group/class of broad-spectrum antimicrobials to be described, dating from 1944 when chlortetracycline was isolated from *Streptococcus aureofaciens*. This compound was introduced four years later, and other agents such as oxytetracycline (from *S. rimosus*) and tetracycline HCl (derived by dehalogenetion of chlortetracycline) were discovered or produced by synthetic processes from 1950-1953. The more commonly used long-acting derivatives, doxycycline and minocycline, were discovered between 1965 and 1972.

These compounds are complex polycycline structures with a carboxamide at position C-2. Substitutions producing the varied agents in the class were generally minimal (tetracycline as base molecule), usually at positions C-5, -6 and -7. Examples: for doxycycline a loss of a hydroxyl group (deoxy) at the C-6ß position, and for minocycline, an addition of a dimethylamino group at position C-7. These chemical alterations change the lipophilicity of the compound with the more hydrophilic agents (tetracycline and oxytetracycline) being least active. Minocycline (most lipophilic) is generally the most potent agent followed by doxycycline. The tetracyclines are usually bacteriostatic, but the MBC may only be 4X MIC higher.

Since the early days of standardized susceptibility testing methods (Clinical and Laboratory Standards Institute [CLSI]; formerly the NCCLS), the testing of tetracycline used a 30-µg tetracycline HCl disk as the class disk. The earliest NCCLS interpretive tables were for the M2-A and M2-A2 standards published before 1980, each table containing only tetracycline HCl interpretive zone diameters (susceptible at ≥19 mm [MIC correlate at ≤4 µg/ml]; resistant at ≤14 mm [MIC correlate at ≥12 µg/ml]). When the annual supplemental table program was initiated in 1981, only tetracycline HCl was listed with a statement (footnote p) that read "tetracycline is the class disk for all tetracyclines, and the results can be applied to chlortetracycline, demeclocycline, doxycycline, methocycline, minocycline and oxytetracycline. However, some in vitro data show that certain organisms may be more susceptible to doxycycline and minocycline than to tetracycline." The following year (1982) in NCCLS M2-A2 S2, interpretive disk diffusion criteria for doxycycline (susceptible at ≥16 µg/ml, resistant at ≤12 mm) and minocycline (susceptible at ≥19 mm, resistant at ≤14 mm) were added to the published tables. These criteria and their MIC correlation have not been altered in more than two decades when these guidelines were adopted based on a single-laboratory study comparing the disk diffusion results for the three tetracycline derivatives.

In subsequent years, the value of minocycline has been recognized in the treatment of methicillin-resistant *Staphylococcus aureus* and resistant *Acinetobacter* spp.; and doxycycline has been successfully applied to the therapy of vancomycin-resistant enterococci. These events necessitated expanded testing of this class and reports of discords between MIC and disk diffusion results. The *Acinetobacter* and Polymyxin Working Group of the CLSI Antimicrobial Susceptibility Testing Subcommittee addressed these concerns via a structured, multicenter comparison of three tetracyclines tested by reference MIC and standardized disk diffusion methods against contemporary strains of Enterobacteriaceae (this report) and *Acinetobacter* spp. (see Tables in M100-S16, 2006).

MATERIALS AND METHODS

Study Design: Each of five participating laboratories was requested to test Enterobacteriaceae isolates by reference broth microdilution and disk diffusion methods according to a common protocol.

Participating Laboratories:
- Center for Disease Control and Prevention (Atlanta, GA);
- Duke University (Durham, NC);
- JMI Laboratories (North Liberty, IA);
- Loyola University Medical Center (Maywood, IL);
- Massachusetts General Hospital (Boston, MA).

Bacteria Isolates: A total of 454 clinical strains were tested against tetracycline and doxycycline, while 288 of those were evaluated against minocycline. The distribution of isolates listed by the species and institution is found in Table 1.

Susceptibility Testing: The isolates were tested for susceptibility against tetracycline, doxycycline, and minocycline by reference frozen-form broth microdilution and disk diffusion methods according to CLSI standards (M2-A9 and M7-A7, 2006). The MIC portion of the study utilized a common lot of panels prepared by TREK Diagnostics (Cleveland, OH); while antimicrobial disks were manufactured by BD Diagnostics (Sparks, MD). Each participant laboratory tested *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 on five occasions as quality control (QC) organisms; all recorded (QC) results were within published ranges (M100-S16, 2006) for presented data.

Breakpoints: Tetracycline, doxycycline and minocycline MIC and disk diffusion breakpoints established for Enterobacteriaceae by the CLSI were applied for all pathogens evaluated in the present study. The broth microdilution and disk diffusion results for each drug were compared by regression and error-rate bounding analyses (M23-A2, 2001). Since tetracycline disk results may be used to predict the susceptibility for doxycycline and minocycline, we also evaluated the correlation between tetracycline disk diffusion inhibition zones and those of doxycycline and minocycline (cross-resistance analysis).

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ACKNOWLEDGEMENT

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RESULTS

- The proposed disk diffusion breakpoints are 2 to 4 mm smaller than the current (CLSI, 2006) zone diameters (Table 2).

Table 1. Distribution of isolates by the institution of origin.

	Number of isolates tested at[a]:				
Organism (no. tested)	CDC	Duke	JMI	MGH	Total
Citrobacter freundii	3	1	43	3	50
Citrobacter koseri	0	1	3	3	7
Enterobacter cloacae	3	4	40	3	50
Enterobacter spp.[b]	3	4	3	1	11
Escherichia coli	10	10	70	10	100
Indole-positive *Proteae*	5	7	9	7	28
Klebsiella oxytoca	0	1	49	0	50
Klebsiella pneumoniae	10	10	70	10	100
Proteus mirabilis	5	4	10	5	24
Salmonella/Shigella[c]	5	0	0	3	8
Serratia spp.[d]	4	5	10	5	24
Others[e]	0	2	0	0	2
Total	48	49	307	50	454

a. CDC: Center for Disease Control and Prevention, Altanta, GA; Duke: Duke University, Durham, NC; JMI: JMI Laboratories, North Liberty, Iowa; MGH: Massachusetts General Hospital, Boston, MA.
b. Includes *Enterobacter aerogenes* (9 strains), *E. asburiae* (one strain), *E. sakazakii* (one strain).
c. Includes *Salmonella enteritidis* (one strain), *S. typhimurium* (two strains), *Salmonella* spp. (one strain), *Shigella sonnei* (one strain) and *Shigella* spp. (three strains).
d. Includes: *Serratia liquifaciens* (one strain) and *S. marcescens* (23 strains).
e. Includes *Hafnia alvei* (one strain) and *Pantoea agglomerans* (one strain).

Table 2. Tetracycline breakpoints for Enterobacteriaceae.

	Breakpoints (mm)			
	CLSI 2006		Proposed (CLSI 2007)	
Antimicrobial agent	Susceptible	Resistant	Susceptible	Resistant
Tetracycline	≥19	≤14	≥15	≤11
Doxycycline	≥16	≤12	≥14	≤10
Minocycline	≥19	≤14	≥16	≤12

Table 3. Summary of error rates.

	Current CLSI breakpoints			Proposed breakpoints		
Antimicrobial	VM	Ma	Mi	VM	Ma	Mi
Tetracycline	0.0%	0.9%	15.0%	0.0%	0.0%	4.6%
Doxycycline	0.0%	0.4%	11.5%	0.0%	0.0%	5.5%
Minocycline	0.0%	0.7%	30.6%	0.3%	0.0%	8.3%

Abbreviations: VM, very major errors (false-susceptibility); Ma, major errors (false-resistance); Mi, minor errors (errors involving intermediate category).

Figure 1. Correlation between tetracycline MIC results and disk diffusion inhibition zones. Solid lanes indicate 2006 CLSI breakpoints while dashed lines indicate the 2007 CLSI disk diffusion breakpoints.

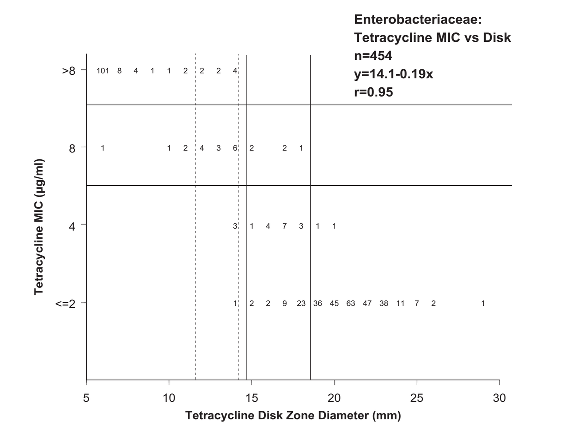


Figure 2. Correlation between doxycycline MIC results and disk diffusion inhibition zones. Solid lanes indicate 2006 CLSI breakpoints while dashed lines indicate the 2007 CLSI disk diffusion breakpoints.

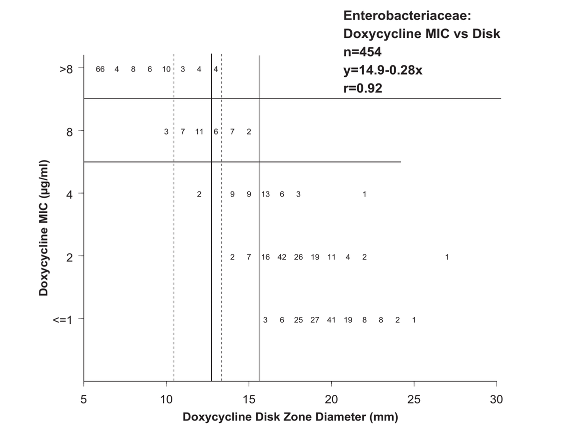


Figure 3. Correlation between minocycline MIC results and disk diffusion inhibition zones. Solid lanes indicate 2006 CLSI breakpoints while dashed lines indicate the 2007 CLSI disk diffusion breakpoints.

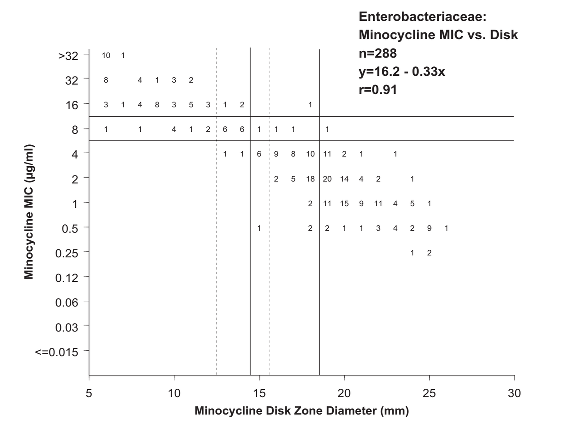
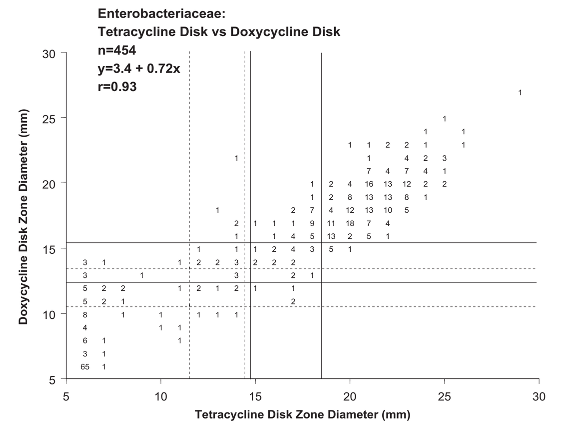


Figure 4. Correlation between tetracycline and doxycycline disk diffusion inhibition zones. Solid lanes indicate 2006 CLSI breakpoints while dashed lines indicate the 2007 CLSI disk diffusion breakpoints.



- Figure 1 shows the correlation between tetracycline MIC and disk diffusion inhibitory zone diameters using both the current (CLSI, 2006) and the proposed breakpoints (CLSI, 2007 [pending]). The adjustment of the breakpoints eliminated the major errors and produced a significant decrease in the rate of minor errors (from 15.0 to 4.6%).

- When testing doxycycline, an adjustment of the disk diffusion breakpoints could also eliminate the major errors and decrease the minor errors from 11.5 to an acceptable 5.5% (Figure 2).

- The minor errors for minocycline were markedly minimized from 30.6 to 8.3% by modifying the disk diffusion zone breakpoints (Figure 3). No major error was observed when using the proposed breakpoints; however, one strain (0.3%; acceptable level) was susceptible by disk diffusion and resistant by broth microdilution method (very major errors).

- With these proposed breakpoints, no major errors were observed and a single very major error was seen only for minocycline. Furthermore, minor error rates varied from only 4.6% for tetracycline to 8.3% for minocycline.

- Tetracycline disk diffusion results can still be used to predict susceptibility for doxycycline (Figure 4) and minocycline using these modified (CLSI, 2007 [pending]) breakpoints. All isolates susceptible to tetracycline were also susceptible to doxycycline (Figure 4), and 98.6% of isolates susceptible to tetracycline were susceptible to minocycline (data not shown).

CONCLUSIONS

- The current CLSI disk diffusion breakpoints (M100-S16, 2006) provide unacceptable rates of interpretive errors when testing tetracycline, doxycycline and minocycline against Enterobacteriaceae, thus requiring modifications.

- The proposed adjustments in the breakpoints provided acceptable intermethod error rates (M23-A2, 2001) for testing Enterobacteriaceae against tetracycline compounds by the disk diffusion method (M2-A9, 2006).

- The tetracycline class breakpoints established based on the results of this study were approved by the CLSI Subcommittee on Antimicrobial Susceptibility in June 2006 and will be published in the 2007 CLSI document M100-S17.