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Comparison of Etest, Disk Diffusion, and Broth Macrodilution for *In Vitro* Susceptibility Testing of *Rhodococcus equi*

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MICs of erythromycin, clarithromycin, azithromycin, rifampin, gentamicin, and doxycycline against 101 isolates of *Rhodococcus equi* were determined by broth macrodilution, disk diffusion, and Etest. Categorical agreement ranged between 85.1 and 100%. Overall, the agreement between Etest and disk diffusion was better than the agreement between broth macrodilution and the agar-based methods.

Rhodococcus equi, a Gram-positive facultative intracellular pathogen, is one of the most important causes of disease in foals between 3 weeks and 5 months of age. *R. equi* has also emerged as a common opportunistic pathogen in immunosuppressed people, especially those infected with the human immunodeficiency virus (1–3). Infection in either species is most commonly characterized by life-threatening pyogranulomatous pneumonia (3, 4).

A wide variety of antimicrobial agents are active against *R. equi in vitro*. However, many of these drugs are reported to be ineffective *in vivo*, likely because of poor cellular uptake and resulting low intracellular concentrations (5). The combination of a macrolide (erythromycin, azithromycin, or clarithromycin) and rifampin has been the mainstay of therapy in foals infected with *R. equi* since the early 1980s (6, 7). Until recently, reports of macrolide resistance have been rare. Over the past decade, there has been an increase in the frequency of detection of macrolide and rifampin resistance in isolates of *R. equi* from pneumonic foals (8), and resistant isolates were cultured from up to 40% of affected foals at a large breeding farm (9). Because foals infected with macrolide and rifampin-resistant isolates are significantly more likely to die (8), it is of paramount importance to obtain accurate *in vitro* susceptibility testing results early in the disease process.

Antimicrobial susceptibility testing can be done using a variety of different methods, with broth dilution, disk diffusion, and Etest being commonly used by veterinary diagnostic laboratories. To date, there are no data comparing the performance of these methods for the determination of *in vitro* activity of antimicrobial agents against *R. equi*. In one study, 12 *R. equi* isolates identified as resistant to azithromycin, clarithromycin, erythromycin, or rifampin in a diagnostic laboratory using disk diffusion were determined to be susceptible to these drugs upon subsequent retesting with a different method (8). Therefore, the objective of this study was to compare the results of broth macrodilution, disk diffusion, and Etest for *in vitro* susceptibility testing of macrolide-susceptible and macrolide-resistant isolates of *R. equi*.

A total of 101 nonduplicate *R. equi* isolates obtained from tracheobronchial aspirates or postmortem lung tissue from pneumonic foals in the United States between March 2000 and January 2011 were used. Twenty isolates were randomly selected from a collection of isolates previously identified as resistant to one or more macrolide antimicrobial agents. The other isolates ($n = 81$) were randomly selected from a collection of frozen stabilates. Isolates were confirmed to be *R. equi* and to contain the virulence plasmid by PCR amplification of the *choE* and *vapA* genes, respectively, as previously described (10). Antimicrobial agents investigated in this study (erythromycin, clarithromycin, azithromycin, rifampin, gentamicin, and doxycycline) were selected based on excellent *in vitro* activity against large numbers of isolates of *R. equi* (11, 12) and frequency of use in foals (5, 7, 13). Reference standards for antimicrobial agents were purchased from U.S. Pharmacopeia (Rockville, MD).

The isolates were removed from storage and subcultured on Trypticase soy agar. Each inoculum was prepared by the direct colony suspension method according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI), resulting in the recommended inoculum of approximately 5×10^5 as verified by CFU counting (14). For a given isolate, susceptibility testing with all 3 methods was performed with the same inoculum. For each isolate, the MIC was determined by a dilution broth macrodilution technique in glass tubes in accordance to the guidelines established by the CLSI (15). Concentrations of antimicrobial agents tested represented 2-fold dilutions between 256 and 0.031 μg/ml. The MIC was defined as the first dilution with no bacterial growth after 24 h of incubation at 35 ± 2°C. Etest strips (BioMérieux, Durham, NC) were used according to the manufacturer's recommendations for each antimicrobial. Concentrations of antimicrobial agents tested represented 2-fold dilutions between 256 and 0.016 μg/ml for all antimicrobial agents, with the exception of rifampin, which had a concentration range between 32 and 0.002 μg/ml. Disk diffusion susceptibility testing was performed using commercially available disks (BD, Franklin Lakes, NJ) in accordance with the CLSI guidelines (14).

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Received 15 September 2014 **Returned for modification** 8 October 2014
Accepted 28 October 2014

Accepted manuscript posted online 5 November 2014

Citation Berghaus LJ, Giguère S, Guldbech K, Warner E, Ugorji U, Berghaus RD. 2015. Comparison of Etest, disk diffusion, and broth macrodilution for *in vitro* susceptibility testing of *Rhodococcus equi*. J Clin Microbiol 53:314–318. doi:10.1128/JCM.02673-14.

Editor: B. W. Fenwick

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doi:10.1128/JCM.02673-14

TABLE 1 Interpretive criteria[a] used for the classification of *R. equi* isolates as susceptible, intermediate, or resistant to azithromycin, clarithromycin, erythromycin, rifampin, gentamicin, and doxycycline

Antimicrobial	Breakpoint					
	Susceptible		Intermediate		Resistant	
	Broth/Etest (μg/ml)	Disk (mm)	Broth/Etest (μg/ml)	Disk (mm)	Broth/Etest (μg/ml)	Disk (mm)
Azithromycin	≤2	≥18	4	14–17	≥8	≤13
Clarithromycin	≤2	≥18	4	14–17	≥8	≤13
Erythromycin	≤0.5	≥23	1–4	14–22	≥8	≤13
Doxycycline	≤4	≥16	8	13–15	≥16	≤12
Gentamicin	≤2	≥16	4	13–15	≥8	≤12
Rifampin	≤1	≥20	2	17–19	≥4	≤16

[a] There are currently no CLSI-approved interpretive criteria for the susceptibility testing of *R. equi*. Thus, CLSI interpretive criteria for *Staphylococcus aureus*, as commonly reported for *R. equi* by many veterinary diagnostic laboratories and widely reported in the literature, were used (8, 9, 16–18).

the susceptibility testing of *R. equi* in people or horses. Thus, CLSI interpretive criteria for *Staphylococcus aureus*, as commonly reported for *R. equi* by many veterinary diagnostic laboratories and widely reported in the literature (8, 9, 16–18), were used for azithromycin, clarithromycin, erythromycin, doxycycline, and rifampin (Table 1). Equine-specific interpretive criteria established by the Subcommittee on Veterinary Antimicrobial Susceptibility Testing of the CLSI for Gram-negative bacteria were used for gentamicin (Table 1) (14). Control strains tested in parallel and on each test occasion for all methods were *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. *R. equi* ATCC 33701, a virulent strain with a known *in vitro* susceptibility profile, was used as an additional control (19).

MIC_{90}s and MIC_{50}s were calculated for the Etest and broth macrodilution for each antimicrobial agent. For each antimicrobial agent, MICs obtained with the Etest were compared to MICs obtained with the broth macrodilution method using the Wilcoxon signed-rank test. For each antimicrobial agent, the results obtained by the 3 methods tested were converted to qualitative categories (Table 1). For a given antimicrobial agent, the percentage of susceptible isolates and the percentage of resistant isolates were compared between the 3 methods using the Cochran Q test for paired proportions. Categorical agreement between qualitative results for two test methods was achieved when an isolate was classified within the same category (i.e., susceptible, intermediate, or resistant) by both testing methods. Categorical discrepancies were recorded as minor (an intermediate result was obtained by only one of the methods compared), major (susceptible isolate misinterpreted as resistant) and very major (resistant isolate misinterpreted as susceptible) errors. The broth macrodilution test was considered the reference method for these determinations except when comparing Etest to disk diffusion, when the Etest was considered the reference method.

Essential agreement between the Etest and broth macrodilution was defined as the percentage of MIC pairs that differed by ± 1 $\log_2$ dilution or less. Bias between the Etest and broth macrodilution was calculated using the method described by Bland and Altman (20) on $\log_2$-transformed MIC data. For each drug, bias was calculated as the mean difference between the $\log_2$ MIC determined using the Etest and the corresponding $\log_2$ MIC determined using broth macrodilution. A positive bias value indicated that the Etest tended to overestimate the MIC, whereas a negative bias value indicated that the Etest tended to underestimate the MIC, compared to the MIC determined via the broth macrodilution. For all analyses, a P value of <0.05 was considered significant.

Quality control data for each of the ATCC reference strains for all antimicrobial agents were within the acceptable range as specified by the CLSI and manufacturers of the Etest and disk diffusion assays. MIC data and percentages of susceptible, intermediate, and resistant isolates for each method are presented in Table 2. The percentage of isolates classified as susceptible to gentamicin (93.1%) by the broth macrodilution method was significantly lower than that obtained with the Etest or the disk susceptibility method (99%) (Table 2). The percentage of isolates classified as resistant to erythromycin was significantly higher with the broth macrodilution method (25.7%) than with the Etest (19.0%), whereas the percentage of resistance to rifampin was significantly lower with broth macrodilution (25.7%) than with disk diffusion (31.7%) (Table 2).

The overall categorical agreement between tests ranged between 85.1 and 100%, with the lowest agreement being for the comparison of the broth macrodilution test with the disk diffusion test for erythromycin and perfect agreement being found for Etest versus the disk diffusion test for gentamicin. At least one very major error (falsely susceptible) was detected in 10 of 18 possible comparisons by antibiotic and test. Major errors (falsely resistant) were detected in 4 of 18 comparisons, and minor errors (all other discrepancies) were detected in 17 of 18 comparisons (Table 3).

Essential agreement between Etest and broth macrodilution ranged between 70.3% for erythromycin and 86.1% for doxycycline (Table 4). For clarithromycin, erythromycin, gentamicin, and doxycycline, the MIC obtained using the broth macrodilution method was significantly different than that obtained with the Etest (Table 4). MICs were not significantly different between the 2 methods for azithromycin and rifampin. Etest tended to overestimate MICs relative to broth macrodilution for clarithromycin and gentamicin and underestimate MICs for erythromycin and doxycycline (Table 4).

The importance of reliable antimicrobial susceptibility testing methods for *R. equi* cannot be overstated given that foals treated with the traditional combination of a macrolide and rifampin despite being infected with macrolide- and rifampin-resistant isolates are approximately 7 times more likely to die than foals infected with susceptible isolates (8). Disk diffusion and Etest were selected for comparison to broth macrodilution in the present study because they are commonly used by veterinary diagnostic laboratories, with the disk diffusion method being the most widely used. There are currently no CLSI-approved interpretive criteria for the susceptibility testing of *R. equi*. However, comparison of the performance of the disk diffusion method relative to that of the broth macrodilution or Etest required classification of the isolates as susceptible, intermediate, or resistant. The present study used the CLSI interpretive criteria for *Staphylococcus aureus* as commonly reported for *R. equi* by many veterinary diagnostic laboratories and widely reported in the literature (16–18). It will be up to the Subcommittee on Veterinary Antimicrobial Susceptibility Testing of the CLSI to determine whether these interpretive criteria are appropriate or not. However, in the interim, there is a need for data regarding the relative performance of suscepti-

TABLE 2 Antimicrobial susceptibilities of 101 isolates of *R. equi* to azithromycin, clarithromycin, erythromycin, rifampin, gentamicin, and doxycycline, determined by broth macrodilution, Etest, and disk diffusion[a]

Antimicrobial	Test	MIC_{90} (μg/ml)	MIC_{50} (μg/ml)	Range (μg/ml)	S (%)	I (%)	R (%)
Azithromycin	Broth	128	1	(0.03–256)	69.3	3.0	27.7
	Etest	32	1.5	(0.016–>256)	74.3	1.0	24.8
	Disk	NA	NA	NA	73.3	2.0	24.8
Clarithromycin	Broth	16	0.125	(<0.03–128)	75.2	3.0	21.8
	Etest	16	0.125	(0.03–>256)	76.2	2.0	21.8
	Disk	NA	NA	NA	77.2	2.0	20.8
Erythromycin	Broth	64	0.5	(0.06–256)	74.3	0	25.7[c]
	Etest	16	0.38	(0.09–>256)	76.0	5.0	19.0
	Disk	NA	NA	NA	74.3	2.0	23.8
Rifampin	Broth	32	0.25	(<0.03–>256)	72.3	2.0	25.7[d]
	Etest	32	0.25	(0.012–>32)	70.3	0	29.7
	Disk	NA	NA	NA	68.3	0	31.7
Gentamicin	Broth	1	0.5	(<0.03–>256)	93.1[b]	4.0	3.0
	Etest	1.5	1	(<0.016–>256)	99.0	0	1.0
	Disk	NA	NA	NA	99.0	0	1.0
Doxycycline	Broth	2	1	(0.03–4)	99.0	0	1.0
	Etest	1.5	0.5	(0.016–32)	99.0	0	1.0
	Disk	NA	NA	NA	98.0	0	2.0

[a] S, susceptible; I, intermediate; R, resistant. NA, not applicable.
[b] Significant difference between the percentage of susceptibility obtained by broth macrodilution and that obtained with the Etest or disk diffusion ($P = 0.002$).
[c] Significant difference between the percentage of resistance obtained by broth macrodilution and that obtained with the Etest ($P = 0.029$).
[d] Significant difference between the percentage of resistance obtained by broth macrodilution and that obtained with disk diffusion ($P = 0.034$).

bility testing methods with the interpretive criteria currently being used clinically.

Approximately 20 to 30% of the isolates tested in this study were resistant to macrolides or rifampin. This number is artificially inflated because 20 isolates were selected purposefully based on high MICs for macrolides and rifampin. In a prior study, the prevalence of *R. equi* isolates resistant to macrolide antimicrobial agents and rifampin in Florida and Texas was approximately 4%

TABLE 3 Agreement between broth macrodilution, Etest, and disk diffusion and percentage of each type of error for 101 isolates of *Rhodococcus equi*

Antimicrobial	Test comparison	Agreement (%)	Very major errors (%)	Major errors (%)	Minor errors (%)
Azithromycin	Broth vs disk	93.1	5.0	0	2.0
	Etest vs disk	98.0	1.0	0	1.0
	Broth vs Etest	93.1	4.0	0	3.0
Clarithromycin	Broth vs disk	95.0	1.0	1.0	3.0
	Etest vs disk	96.0	1.0	1.0	2.0
	Broth - Etest	97.0	0	0	3.0
Erythromycin	Broth vs disk	85.1	4.0	1.0	9.9
	Etest vs disk	92.1	1.0	1.0	5.9
	Broth vs Etest	89.1	3.0	0	7.9
Rifampin	Broth vs disk	99.0	0	0	1.0
	Etest vs disk	98.0	0	0	2.0
	Broth vs Etest	99.0	0	0	1.0
Gentamicin	Broth vs disk	94.1	2.0	0	4.0
	Etest vs disk	100	0	0	0
	Broth vs Etest	94.1	2.0	0	4.0
Doxycycline	Broth vs disk	94.1	0	0	5.9
	Etest vs disk	99.0	0	0	1.0
	Broth vs Etest	93.1	0	0	6.9

TABLE 4 Comparison of MIC results obtained by Etest and the broth macrodilution method for 6 antimicrobial agents against 101 isolates of *R. equi*

Antimicrobial	No. of isolates for which Etest MIC differed from broth macrodilution MIC by given no. of $\log_2$ dilutions							Essential agreement (%)	Bias (95% CI)[a]
	≤−3	−2	−1	0	1	2	≥3		
Azithromycin	8	7	10	44	21	9	2	74.3	−0.01 (−0.32 to 0.29)
Clarithromycin	4	0	9	40	34	10	4	82.2	0.53 (0.25 to 0.80)[b]
Erythromycin	11	12	19	44	8	2	4	70.3	−0.67 (−0.99 to −0.35)[a]
Rifampin	6	4	12	58	14	4	3	83.2	−0.09 (−0.49 to 0.30)
Gentamicin	2	6	7	42	26	4	14	74.3	0.84 (0.47 to 1.21)[b]
Doxycycline	3	6	30	46	11	3	2	86.1	−0.39 (−0.67 to −0.13)[b]

[a] CI, confidence interval.
[b] Significant difference between broth macrodilution and Etest MIC ($P < 0.05$).

(8). However, up to 40% of the *R. equi* isolates at one farm in Kentucky were found to be resistant to these antimicrobial agents (9). As documented with a smaller number of isolates in prior studies (8, 19), isolates of *R. equi* resistant to macrolides and rifampin in the present study were typically susceptible to gentamicin and doxycycline *in vitro*. Because almost all isolates were susceptible to gentamicin and doxycycline, the present study was less likely to detect errors in categorical agreement for these antimicrobial agents.

The present study demonstrated that the Etest tended to slightly underestimate MICs for erythromycin and doxycycline and to slightly overestimate MICs for clarithromycin and gentamicin relative to results with broth macrodilution. Overall, 70.4 to 86.1% of the paired MICs were within 1 $\log_2$ dilution. However, 5 to 15% of the MIC pairs were more than 2 $\log_2$ dilutions apart. This discrepancy is more than expected based on studies comparing Etest to broth dilution testing for various microorganisms and antimicrobial agents (21–23). Despite sometimes marked differences in actual MICs, the categorical agreement between the 2 quantitative methods was better, ranging from 89.1% for erythromycin to 99.0% for rifampin. This indicated that most discrepancies in MICs did not impair classification of *R. equi* as susceptible, intermediate, or resistant. The proportion of very major errors was outside the target accuracy used by the U.S. Food and Drug Administration (24) for azithromycin and erythromycin in this study. It is unclear if these discrepancies reflect a higher rate of errors with the Etest, broth macrodilution, or a combination of both. Overall, the agreement between Etest and disk diffusion was better than the agreement between broth macrodilution and either agar-based method. These results suggest that broth macrodilution may not be a perfect gold standard to which other methods are being compared. Broth macrodilution was selected for use in this study because commercially available microtiter plates have a very limited range of dilutions which would have prevented quantitative comparisons to the Etest over the entire range of possible MICs. There are multiple possible source for mistakes with the broth macrodilution method, and difficulties in reading broth dilution assays at 24 h for some isolates of *R. equi* have been reported previously (25). A recent publication demonstrated that addition of 2% (vol/vol) lysed horse blood to the cation-adjusted Mueller-Hinton broth facilitates the discrimination between growth and no growth of *R. equi* (26). Lysed horse blood was not used in the present study because this information was not available at the time this study was conducted. Additional studies will be necessary to determine if addition of lysed horse blood improves the agreement between broth macrodilution and Etest.

In conclusion, the present study documented important discrepancies in the results of *in vitro* susceptibility testing of *R. equi* depending on the method used. These data demonstrate the need for harmonization of antimicrobial susceptibility testing methods for *R. equi* and establishment of *R. equi*-specific interpretive criteria.

ACKNOWLEDGMENTS

This work was funded by the Hodgson Equine Research Endowment of the University of Georgia.

We thank veterinarians and diagnostic laboratories who provided isolates.

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