Colorimetric-Plate Method for Rapid Disk Diffusion Susceptibility Testing of *Escherichia coli*[▽]

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Here, we report a laboratory-developed colorimetric-plate method for rapid disk diffusion susceptibility testing of *Escherichia coli*. One hundred isolates were evaluated. Categorical agreement between the colorimetric plate and the standard disk diffusion method was 99%. Mean time to results was 7.07 h (95% confidence interval, 5.96 to 8.19).

Escherichia coli is the most frequent cause of urinary tract infections and among the leading pathogens causing bloodstream infections (10, 31). High levels of antibiotic resistance are observed in *E. coli* (6, 13, 29, 31). Severe adverse outcomes such as increased length of hospital stay and delay of appropriate therapy are shown to be associated with multidrug-resistant *Enterobacteriaceae* (26, 30). The overall cost of antibiotic-resistant urinary tract infections to the health care system is high (2, 23). Appropriate antibiotic use is a priority (24, 25, 28, 32). Inappropriate initial antimicrobial therapy is independently associated with adverse outcomes, especially when not adjusted quickly with rapid susceptibility test results (17, 18, 20). Thus, a rapid, simple, and inexpensive alternative to commercial and higher-technology platforms for the susceptibility testing of *E. coli* isolates from urinary specimens is needed (25, 28, 32). Typically, standard antimicrobial susceptibility test methods require 16 to 18 h of incubation to produce interpretable results (7, 9). For rapid susceptibility testing in daily laboratory work, there are several automated systems, providing results within 8 to 12 h (27). Noncommercial rapid colorimetric assays employing oxidation-reduction indicators for antimicrobial susceptibility testing with MICs are described for various bacteria (1). A commercial rapid susceptibility testing medium for disk diffusion susceptibility testing is also reported (19). So far, all of the studies have reported either MIC-adapted methods or commercial disk diffusion methods.

TABLE 1. Reproducibility results of the colorimetric-plate method in antimicrobial susceptibility testing of *Escherichia coli*

Antimicrobial[a]	No. of isolates[b]	No. of tests[c]	No. of test results in interpretive category[e]:			Reproducibility result, % (no.)[d]			
			S	I	R	CA	VMD	MD	mD
Ampicillin	10	270	108	0	162	100	0	0	0
Ampicillin-sulbactam	10	270	169	47	54	97.4	0	0	2.6 (7)
Cefazolin	10	270	162	0	108	100	0	0	0
Cefuroxime	10	270	162	0	108	100	0	0	0
Ceftriaxone	10	270	163	27	80	99.6	~1.3 (1)	0	0
Cefepime	10	270	270	0	0	100	0	0	0
Imipenem	10	270	270	0	0	100	0	0	0
Amikacin	10	270	216	2	52	99.3	0	0	0.7 (2)
Gentamicin	10	270	192	50	28	98.5	0	0	1.5 (4)
Ciprofloxacin	10	270	189	0	81	100	0	0	0
Co-trimoxazole	10	270	162	0	108	100	0	0	0
Nitrofurantoin	10	189	189	0	0	100	0	0	0
All antimicrobials	10	3,159	2,252	126	781	~99.5	0.1 (1)	0	0.4 (13)

[a] Nitrofurantoin was not tested in isolates of nonurine origin.
[b] *E. coli* ATCC 25922 (of nonurine origin) and nine clinical *E. coli* isolates, of which two were of nonurine origin.
[c] Isolates tested in three replicate tests at three distinct sites in three different days.
[d] Reproducibility was acceptable if the results showed ≥95% category agreement compared to the test mode (i.e., the most frequent test result for the isolate) as a reference. CA, categorical agreement; VMD, very major discrepancy; MD, major discrepancy; mD, minor discrepancy.
[e] S, susceptible; I, intermediate; R, resistant.

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TABLE 2. Comparative performance metrics of colorimetric-plate method in susceptibility testing of *Escherichia coli*[d]

Antimicrobial[a]	Test method	No. of isolates[b]	No. of tests[a,b]	No. of test results in interpretive category:			Performance analysis result, % (no.)[c]		
				S	I	R	CA	VMD, MD	mD
Ampicillin	CLSI	100	100	5	1	94			
	CP	100	100	5	0	95	99	0	1 (1)
Ampicillin-sulbactam	CLSI	100	100	38	15	47			
	CP	100	100	40	12	48	97	0	3 (3)
Cefazolin	CLSI	100	100	50	1	49			
	CP	100	100	50	4	46	97	0	3 (3)
Cefuroxime	CLSI	100	100	54	0	46			
	CP	100	100	54	0	46	100	0	0
Ceftriaxone	CLSI	100	100	53	4	43			
	CP	100	100	53	5	42	99	0	1 (1)
Cefepime	CLSI	100	100	89	8	3			
	CP	100	100	90	7	3	99	0	1 (1)
Imipenem	CLSI	100	100	100	0	0			
	CP	100	100	100	0	0	100	0	0
Amikacin	CLSI	100	100	97	0	3			
	CP	100	100	96	1	3	99	0	1 (1)
Gentamicin	CLSI	100	100	65	0	35			
	CP	100	100	65	0	35	100	0	0
Ciprofloxacin	CLSI	100	100	43	1	56			
	CP	100	100	43	1	56	100	0	0
Co-trimoxazole	CLSI	100	100	41	0	59			
	CP	100	100	41	0	59	100	0	0
Nitrofurantoin	CLSI	68	68	61	5	2			
	CP	68	68	61	5	2	100	0	0
All antimicrobials	CLSI	100[a]	1,168[a,b]	696	35	437			
	CP	100[a]	1,168[a,b]	698	35	435	~99.1	0	~0.9 (10)
FDA criteria[c]							≥90	≤1.5, ≤3	≤5.5–10[c]

[a] Nitrofurantoin was not tested in isolates of nonurine origin.

[b] Not including the quality control strain *E. coli* ATCC 25922.

[c] FDA definitions, formulae, and acceptable performance criteria were used in performance analysis. Acceptable performance criteria for mD rate as an acceptable criterion or improved criterion were defined for the purposes of this study by the authors.

[d] Abbreviations: CLSI, standard disk diffusion method of the Clinical and Laboratory Standards Institute; CP, colorimetric-plate method; S, susceptible; I, intermediate; R, resistant; CA, categorical agreement; VMD, very major discrepancy; MD, major discrepancy; mD, minor discrepancy; FDA, Food and Drug Administration.

However, a simpler or cheaper alternative to commercial and higher-technology platforms does not exist. Such an alternative is much needed, especially for the susceptibility testing of *E. coli* isolates from the urinary specimens of outpatients, because the isolates constitute most of the daily laboratory workload, and conventional abbreviated identification of *E. coli* is easy, cheap, and sufficient (8). In this study, we have developed a rapid colorimetric medium for disk diffusion susceptibility testing of *E. coli*.

The study was conducted on nonrepetitive clinical isolates (*n* = 100) of *E. coli*. The study collection was characterized with conventional tests and the Vitek 2 (bioMérieux, France) automated microbiology system. Sixty-eight strains were isolated from urine samples, and 28 were extended-spectrum beta-

lactamase (ESBL) producers. For the preparation of colorimetric plates, resazurin additive reagent was freshly prepared by dissolving 2.5 mg of resazurin sodium salt (Sigma-Aldrich, Germany) in 10 ml distilled water and sterilized using a syringe with an 0.2-μm filter (Schleicher & Schuell, Germany). Modified Mueller-Hinton agar medium was prepared by adding 10 ml of resazurin reagent to 990 ml of freshly prepared and cooled (45 to 50°C) Mueller-Hinton agar (Merck, Germany) medium (pH 7.0). Colorimetric plates were prepared by pouring ca. 70 ml of the modified Mueller-Hinton agar medium into each of the 150-mm by 15-mm bacteriological petri dishes to obtain a 4-mm depth. The plates were protected from light. When not used the same day, plates were stored at 4°C for up to 7 days. The procedure described by the Clinical and Labo-

ratory Standards Institute (CLSI) standard was employed for performing the disk diffusion susceptibility tests by the colorimetric-plate method (9). All isolates were also tested with the standard method (9). Performance standards defined in the CLSI standard were used in the interpretation of test results (7). The color change of the medium from blue (resazurin) to pink (resorufin) was observed with the unaided eye. From the start of the incubation period, the observations were done at hourly intervals up to 5 h and then at 10-min intervals. When a visible color change was observed, the completed period was recorded as time to results (i.e., hours). The results of the experiments were valid if the results obtained with the quality control strain were within the acceptable limits given in the CLSI standard and there were no growth and color change in the negative-control plate after the incubation period (7). For reproducibility testing, 10 organisms were tested with the new method, at three distinct laboratory sites (by different personnel on each site) on three separate days in triplicate with a different inoculum prepared for each test (16). Reproducibility results were acceptable if the results of the overall reproducibility study from all test sites for any antimicrobial agent showed ≥95% category agreement compared to the test mode (i.e., the most frequent test result for the isolate) as a reference (16). Acceptable performance criteria were as follows: category agreement, ≥90%; very major discrepancy, ≤1.5%; major discrepancy, ≤3%; minor discrepancy, ≤10% (16). Performance metrics of the new method in terms of time to results were reported as the mean value with 95% confidence limits. The kappa statistic was calculated to measure agreement between the new method and the reference method. The differences between mean times to results of fresh and stock isolates were compared by the Student t test. The significance level for statistical tests was accepted as $P < 0.05$.

Results of the reproducibility tests were evaluated in comparison to the test mode as a reference (Table 1). Overall reproducibility was ca. 99.5%. There was no very major or major discrepancy between the results of the colorimetric-plate method and the standard method (Table 2). Overall category agreement of the colorimetric-plate method was ca. 99%. There was very good agreement between the standard and the new method as shown by the kappa statistic ($\kappa = 0.983$; $P < 0.001$). Times to results were determined by the observation of a visible color change (Fig. 1). Overall mean time to results was 7.07 h (95% confidence interval, 5.96 to 8.19). Mean times to results were 6.25 h (95% confidence interval, 5.23 to 7.27) for fresh clinical isolates and 8.17 h (95% confidence interval, 6.28 to 10.06) ($P = 0.015$) for clinical stock isolates.

For rapid antimicrobial susceptibility testing in daily laboratory work, there are various methods already available. One approach is using automated antimicrobial susceptibility test systems, providing results within 8 to 12 h, with proven performance and the added value of integrated data analysis and expert system utilities (3, 4, 11, 12, 27). As a noncommercial alternative, the in-house colorimetric-plate method produced results in 7 h with a minor extra cost to Mueller-Hinton agar medium. Additionally, the colorimetric-plate method has the advantage of potentially being used in gradient-based MIC detection methods on agar media, such as gradient plate, gradient strip, or agar dilution methods. Unlike automated antimicrobial susceptibility test systems, the colorimetric-plate
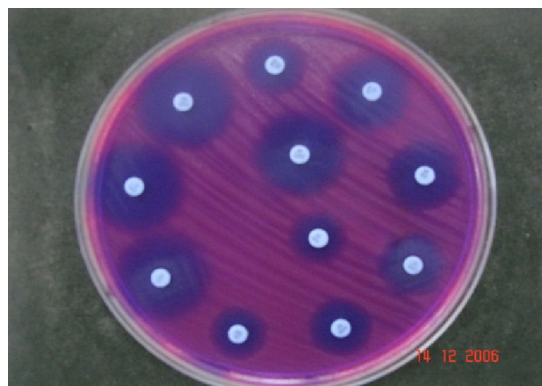

FIG. 1. View of the in-house colorimetric plate for susceptibility testing of *E. coli* after an incubation period of 7 h. Due to the redox indicator resazurin, a background color change from blue (resazurin) to pink (resorufin) is observed in the zones of the plate where *E. coli* colonies grow.

method should also be usable in rapid synergy testing by the gradient strip method on agar media. However, such promising uses of the colorimetric-plate method require further research and evidence. There are also various automated zone reader platforms, at least one of which can be used with a commercially developed rapid disk diffusion susceptibility testing medium providing first results within 4 to 7 h (22). Recently, a rapid susceptibility testing medium for disk diffusion susceptibility testing was reported (19). This system is only commercially available. Previously, various other noncommercial rapid colorimetric assays employing oxidation-reduction indicators for antimicrobial susceptibility testing were described for various bacteria (1). However, MIC-based methods are technically demanding to prepare and perform and are not suitable for daily use. There are also chromogenic media commercially developed for the rapid detection of ESBL-producing organisms (14, 15).

In conclusion, the in-house colorimetric-plate method produced disk diffusion susceptibility test results rapidly and accurately in clinical *E. coli* isolates. However, the interlaboratory reproducibility of the colorimetric-plate method should be evaluated in the future. Further research should focus on analyzing the performance of the colorimetric-plate method in rapid ESBL detection and in rapid susceptibility testing of other important Gram-negative pathogens with an abbreviated identification procedure, potential use in rapid synergy testing, and clinical and economic impact in patient care (5, 11, 21).

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