ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1975, p. 610-611
Vol. 8, No. 5
Copyright © 1975 American Society for Microbiology
Printed in U.S.A.

Microdilution Technique for Antimicrobial Susceptibility Testing of *Haemophilus influenzae*

JAMES H. JORGENSEN* AND JEAN C. LEE

Departments of Pathology and Microbiology, The University of Texas Health Science Center at San Antonio and the Bexar County Hospitals, San Antonio, Texas 78284

Received for publication 25 July 1975

A microdilution method incorporating the use of color-defined growth end points was compared with a conventional broth tube dilution procedure for susceptibility testing of *Haemophilus influenzae* with ampicillin and chloramphenicol. The microdilution method allowed rapid performance of dilution susceptibility tests with easily defined end points.

The existence of ampicillin-resistant strains of *Haemophilus influenzae* has now been well documented in widely separated geographic areas (Morbid. Mortal. Weekly Rep., vol. 24, no. 24, 14 June 1975). This emergence of resistant strains of *Haemophilus* has emphasized the need for practical, reliable susceptibility testing methods for this organism (2, 5, 6).

We have recently reported a simplified medium developed in our laboratory for performance of susceptibility testing of *H. influenzae* (1). This medium is composed of Mueller-Hinton medium plus a yeast concentrate with hematin (Difco supplement C). The advantage of supplement C as an enrichment for growth of *Haemophilus* is that the same basic light color and clarity of Mueller-Hinton medium is retained after the addition of the supplement.

The present report describes a further development of this medium that allows use of rapid microdilution techniques for broth dilution testing of *H. influenzae*. The addition of dextrose and a pH indicator to the basic medium allows the definition of growth end points based on a change in color of the pH indicator.

Seventy-five isolates of *Haemophilus* (70 *H. influenzae* and 5 *H. parainfluenzae*) were utilized to evaluate the microdilution technique. Fifty of these were ampicillin susceptible, whereas twenty-five isolates were known to be resistant to ampicillin (minimal inhibitory concentration [MIC] $\geq$ 8 μg/ml), as confirmed by the Center for Disease Control, Atlanta, Ga.

All isolates were tested for susceptibility to ampicillin and chloramphenicol in parallel by using the traditional broth tube dilution technique and the modified microdilution procedure. The medium used for the conventional tube dilution method was Mueller-Hinton broth plus 5% supplement C (1). For the microdilution test, the medium was composed of: Mueller-Hinton medium, 21 g; dextrose, 10 g; phenol red, 0.036 g; supplement C, 50 ml; and distilled water, 1,000 ml. In practice, the medium is prepared double strength since the addition of the inoculum suspension dilutes the contents of each microtiter well 1:2. Fifty-microliter portions of the two times concentrated medium were added to wells of Falcon sterile, disposable, flat-bottom microtiter plates. Twofold dilutions of ampicillin and chloramphenicol were then prepared in the microtiter plates with flame-sterilized, 50-μl microdiluters. Test concentrations of ampicillin and chloramphenicol included dilutions from 16 to 0.03 μg/ml for the susceptible strains. Those found to be resistant to 16 μg/ml or greater were repeated, starting with ampicillin concentrations of 64 μg/ml. Two wells of each column of the microtiter tray contained only medium, without antibiotic, one of which was inoculated as an organism control, the other left uninoculated as a medium control.

Inocula for both broth dilution methods were prepared by growing *Haemophilus* strains for 4 to 6 h in Mueller-Hinton broth with 5% supplement C. The cell density was adjusted by dilution in sterile 0.9% saline to obtain a turbidity equivalent to that of the 0.5 no. 1 McFarland opacity standard. For conventional tube dilution testing, this inoculum suspension was further diluted 1:100 in saline, and a 0.05-ml portion was added to 0.5 ml of broth. The standard inoculum suspension was diluted 1:500 for the microdilution method, and a 0.05-ml portion was added to the same volume of media in the microtiter wells. The final inoculum density was approximately 2×10^5 organisms/ml with both methods.

Both test procedures included incubation at 37 C for 20 to 24 h without increased CO_2 tension. The MIC for the conventional tube dilu-

TABLE 1. *MIC's of 75 Haemophilus isolates*

Determination	No. and % of isolates with MIC (μg/ml) of:										
	0.125	0.25	0.5	1	2	4	8	16	32	64	>64
Ampicillin (tubes)	19 (13)	26 (35)	14 (19)				1 (1)	1 (1)	3 (4)	11 (15)	9 (12)
Ampicillin (microtiter)		21 (28)	21 (28)	8 (11)			2 (3)	1 (1)	8 (11)	7 (9)	7 (9)
Chloramphenicol (tubes)	1 (1)	10 (13)	47 (63)	16 (22)	1 (1)						
Chloramphenicol (microtiter)	1 (1)	3 (4)	33 (44)	36 (48)	2 (3)						

tion procedure was considered the lowest concentration of antibiotic inhibiting visible growth of the organisms, as determined by turbidity. After incubation of the microtiter tests, growth end points were clearly defined and easily interpreted. The change in the indicator color from red to yellow indicated growth of *Haemophilus* with dextrose fermentation and subsequent acid production. Therefore, the MIC was defined as the lowest dilution of the antibiotic inhibiting visible growth, i.e., the last red well.

The composite results of paired susceptibility tests using the microdilution technique and traditional broth tube dilution method are indicated in Table 1. Results of tests with both antimicrobics indicated that the two test methods agreed within one twofold dilution in all cases. The median concentration of ampicillin that inhibited ampicillin-susceptible isolates was 0.25 μg/ml. Ampicillin MICs of resistant isolates were 8 μg/ml or greater, with a median of 64 μg/ml for the conventional tube dilution procedure and 32 μg/ml for the microdilution method. The median concentration of chloramphenicol that inhibited both groups of organisms was 0.5 μg/ml with the conventional tubes and 1.0 μg/ml for the microtiter method.

A noteworthy observation was that both traditional tube dilution and microdilution tests could be read with confidence as early as 16 h with all strains tested with chloramphenicol. The same statement could be made with ampicillin testing of ampicillin-susceptible isolates. Conversely, the ampicillin MICs of resistant isolates were seen to change as much as four dilutions between 16 and 24 h. For this reason, all final test readings were made between 20 and 24 h.

The need for an efficacious method for the determination of ampicillin susceptibility with *H. influenzae* has been emphasized recently. Several methods and media have been proposed for this purpose, but none has proven entirely satisfactory (3, 4). We have recently described a medium (Mueller-Hinton plus supplement C) that appears suitable for this purpose (1). The present report describes a further modification of this medium that allows rapid broth dilution testing using a microdilution procedure. Microtiter plates, including medium and completed dilutions of antibiotics, may be prepared in advance and stored at −20 C for at least 2 weeks prior to use. Automated devices now available for rapid mechanized pipeting and diluting of fluids in microtiter plates allow performance of broth dilution tests with several strains and different antibiotics in a convenient period of time.

(This paper was presented in part at the 75th Annual Meeting of the American Society for Microbiology, New York, 27 April—2 May 1975.)

LITERATURE CITED

1. Jorgensen, J. H., and P. M. Jones. 1975. Simplified medium for ampicillin susceptibility testing of *Haemophilus influenzae*. Antimicrob. Agents Chemother. 7:186–190.
2. Khan, W., S. Ross, W. Rodriquez, G. Controni, and A. K. Saz. 1974. *Haemophilus influenzae* type B resistant to ampicillin. J. Am. Med. Assoc. 229:298–301.
3. McLinn, S. E., J. D. Nelson, and K. C. Haltalin. 1970. Antimicrobial susceptibility of *Haemophilus influenzae*. Pediatrics 45:827–838.
4. Sell, S. H. W. 1967. *In vitro* sensitivity studies of *Haemophilus influenzae*—typable and non-typable strains. Pediatrics 39:214–219.
5. Thornesberry, C., and L. A. Kirven. Antimicrobial susceptibility of *Haemophilus influenzae*. Antimicrob. Agents Chemother. 6:620–624.
6. Tomeh, M. O., S. E. Starr, J. E. McGowan, Jr., P. M. Terry, and A. J. Nahmias. 1974. Ampicillin-resistant *Haemophilus influenzae* type B infection. J. Am. Med. Assoc. 229:295–297.