A PENICILLINASE TEST INCORPORATED IN THE RAPID CARBOHYDRATE FERMENTATION METHOD FOR RAPID DETECTION OF β-LACTAMASE PRODUCING *NEISSERIA GONORRHOEAE*

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INTRODUCTION

First isolated in 1976 (Ashford *et al.*, 1976; Phillips, 1976), the number of penicillinase-(β-lactamase) producing strains of *Neisseria gonorrhoeae* have since been increasing in many parts of the world, including Asia (Perine *et al.*, 1979). Rockhill *et al.*, (1981) reported the first Penicillinase producing-*N. gonorrhoeae* (PPNG) strains isolated from female patients in West Jakarta, Indonesia. Although prevalence was low, it was evident that strains of PPNG were beginning to emerge in Jakarta, since no PPNG isolates were detected during a previous study (Cobet, 1977-1978, unpublished data).

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To monitor the spread of the PPNG, several rapid methods for detecting β-lactamase production have been developed, including the iodometric (Catlin, 1975), acidometric (Rosen *et al.*, 1972; Thornsberry and Kirven, 1974) and chromogenic cephalosporin tests (O'Callaghan *et al.*, 1972). The acidometric test is based on the color change of phenol red, a pH indicator, produced when penicillin is broken down to penicilloic acid. We made a modification of this test and incorporated it into the rapid carbohydrate fermentation method. We found it was as specific as the iodometric method in detecting β-lactamase. Since that time it has become an integrated part of the routine procedure for *N. gonorrhoeae* identification in our laboratory. This method has greatly simplified the work required for PPNG screening.

The suitability of incorporating the phenol red-penicillinase-test in the rapid fermentation method (PRPT-RF) was confirmed by using the iodometric and penicillin disk diffusion susceptibility methods. These findings are reported herein.

MATERIALS AND METHODS

Bacterial cultures: Fresh clinical isolates and stock cultures of isolates maintained at −70 ° C in trypticase soy broth with 20% (v/v) glycerine, consisted of *N. gonorrhoea*, *N. meningitidis* and *N. lactamica*, from patients with history of gonorrhoeal urethritis, were studied. The clinical isolates were cultured on Modified-Thayer-Martin (MTM) medium and incubated at 35 °C for 24−48 hours in a candle-extinction jar. Identification was made by the Gram strain, oxidase-catalase reaction and the modified rapid fermentation test for carbohydrate utilization pattern (Brown, 1974).

The stock cultures in trypticase soy broth with 20% glycerine frozen at −70 ° C were thawed at room temperature when needed and inoculated onto chocolate agar. A Gram stain was performed to check morphology and purity.

Reagents for rapid carbohydrate fermentation: Buffered salt solution with phenol red indicator (PRBS) was prepared according to Kellogg and Turner (1973) with a minor modification of the phenol red indicator concentration and the pH. One ml of 1% phenol red (aqueous solution) was added to 99 ml of the phosphate buffered salt solution at pH 7.2. Kellogg and Turner (1973) and Brown (1974) used 0.2 ml of 1% phenol red in 100 ml buffer.

Concentrated solutions (20%) of glucose, maltose, lactose, sucrose and fructose were prepared in distilled water, filter sterilized and kept at −20 ° C in 5 ml aliquots until needed. Once thawed, they were stored at 2−6 ° C.

Penicillin solution for phenol red-penicillinase test: Potassium penicillin−G powder (Sigma Chemical Co., St. Louis, Mo.) was dissolved in phosphate buffered salt solution (PBS) pH 7.2 to a final concentration of 6000 µg/ml. The buffered salt solution was the same as used in the rapid fermentation test (Kellogg and Turner, 1973; Brown, 1974) except the phenol red was omitted. The penicillin solution was filter sterilized using a 0.45 µ Swinnex filter (Millipore Corp, Bedford, Mass.). Five ml was dispensed into sterile screw cap vials and stored at −20°C.

Test procedure: The rapid fermentation test was performed according to the method described by Brown (1974). Six tubes (10 × 75 mm), five for carbohydrate fermentation and one for the penicillinase test, were required for each culture. Each tube was labeled appropriately and contained 0.1 ml of PRBS. Two loopfuls (using 3 mm diameter loop) from a 24 hour culture grown on chocolate agar were suspended in 0.35 ml of PRBS. The cell suspension was homogenized with a Vortex mixer (Scientific Industries Inc., Springfield, Mass.) or by aspiration mixing with a Pasteur capillary pipette.

One drop (50 µl) of each carbohydrate solution (glucose, maltose, lactose, sucrose and fructose) was added to the first five tubes appropriately labeled for each carbohydrate. A drop of penicillin−G solution was added to the sixth tube. One drop of the cell suspension was added to each tube and the tubes were then shaken by hand. All tubes were incubated in a 37 ° C waterbath and read after 1−4 hours. The change of color from red to a distinct yellow color in the penicillin tube indicated the presence of β−lactamase. A red or pink color was considered negative for the presence of β−lactamase.

Iodometric test: The rapid iodometric test (Catlin, 1975; Department of Health, Education and Welfare, Public Health Service, Center for Disease Control, Atlanta,

GA., 1976) was used as the standard method to detect β-lactamase.

Disk diffusion susceptibility test: Growth from a 24 hour culture was suspended in Trypticase soy broth, homogenized with a Vortex mixer and adjusted to a 0.5 MacFarland standard. A sterile cotton swab was used to inoculate a chocolate agar plate by the method of Bauer and Kirby (1966). The plate was allowed to dry for approximately 15 minutes, and a 10 unit penicillin disk (BBL, Cockeysville, MD) was placed on the surface. The plate was incubated at 35°C in a candle extinction jar for 18-24 hours. The diameter of the zone of inhibition was measured with a caliper or metric ruler. A zone less than 20 mm indicated resistance to penicillin and a zone of greater than or equal to 20 mm indicated an organism that was sensitive (Department of Health, Education and Welfare, Public Health Service, Center for Disease Control, Atlanta, GA., 1976).

RESULTS

A total of 279 *N. gonorrhoeae* isolates were tested for β-lactamase (Table 1). Of those, 55 were positive for glucose fermentation and penicillinase production by both the PRPT-RF and iodometric methods. These 55 isolates also were resistant to penicillin by the disk diffusion method. The remaining 224 isolates were penicillinase negative by both detection methods; however, 13 of these were resistant to penicillin by the disk diffusion method.

None of 87 *N. meningitidis* and 29 *N. lactamica* cultures produced β-lactamase. They were also sensitive to penicillin by the disk diffusion method.

DISCUSSION

Results of the PRPT-RF test agreed with those given by the iodometric method. These findings were confirmed by the demonstration of penicillin resistance by the disk diffusion method. Although several rapid tests for detecting β-lactamase have been developed (Catlin, 1975; O'Callaghan *et al.*, 1972; Rosen *et al.*, 1972; Thornsberry and Kirven, 1974) and are very useful for monitoring PPNG strains, all are methods separate from the identification procedures for *N. gonorrhoeae*. Compared to our method, extra work and reagents are required to achieve the same results.

We modified the acidometric test (Dhew, 1976); Rosen *et al.*, 1972; Thornsberry and Kirven, 1974), taking the concentration of the penicillin solution (6000 μg/ml) from the iodometric method (Catlin, 1975; Department of Health, Education and Welfare, Public Health Service, Center for Disease Control, Atlanta, GA., 1976; Catlin, 1975) and the reagents to dissolve the penicillin (the buffer salt solution) from the rapid fermentation method (Brown, 1974; Kellogg and Turner, 1973). We then incorporated these into the rapid fermentation system for *Neisseria* sp. (PPNG) identification. Except for the penicillin itself, all the reagents needed to perform the test (the buffer salt solutions for dissolving penicillin and for making the cell suspension) were the same as for the rapid fermentation test.

The reading of the test was done according to the time recommended for the rapid fermentation (1-4 hours) (Brown, 1974). Most of the PPNG strains tested caused a change of the phenol red indicator in the penicillin-tube from red to yellow within 45 seconds to 2 minutes. Some of the strains (less than 7%) failed to show a yellow color until 5-6 minutes of incubation. This might be caused by a smaller amount of β-lactamase production than found in the

Table 1

Results of the PRPT−RF* test of *N. gonorrhoeae*, *N. meningitidis* and *N. lactamica* compared to the iodometric and disk diffusion susceptibility tests.

		Number of strains			
	No.	Positive β-lactamase		Disk diffusion test	
Isolate	tested	PRPT−RF	Iodometric	R (<20 mm)	S (>20 mm)
N. gonorrhoeae	55	55	55	55**	0
	224	0	0	13	211
N. meningitidis	87	0	0	0	87
N. lactamica	29	0	0	0	29

* PRPT−RF = Phenol red-penicillinase test incorporated in the rapid fermentation method.
** Completely resistant, no zone of inhibition.

other strains. There were 13/224 (6%) *N. gonorrhoeae* isolates that were penicillin resistant by the disk diffusion method, but these strains were not β−lactamase producing (non-PPNG) since both the iodometric and PRPT−RF tests were negative. Inadequate inoculum may prolong reaction time, so we recommend reading the PRPT−RF test after 15 minutes. Our experience shows that with this procedure the color change remained stable for a few hours but not after overnight incubation.

SUMMARY

A modification of the acidometric (phenol red) test for penicillinase producing *N. gonorrhoeae* was incorporated into the rapid fermentation method for rapid screening and identification of PPNG strains. Two hundred and twenty-four non-penicillinase-producing *N. gonorrhoeae*, 55 penicillinase-producing *N. gonorrhoeae*, 87 *N. meningitidis* and 89 *N. lactamica* were included in this study. Results of the modified test were comparable with the iodometric and penicillin disk diffusion susceptibility and were obtainable within 1 to 5 minutes.

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