Feasibility of Screening for Penicillinase-Producing *Neisseria gonorrhoeae* from Primary Culture Plates by Using a Rapid Microacidometric Test

ALICE SCHAUER WEISSFELD,¹ GAIL D. SANNER,² JACK R. CHILDRESS,¹ JOHN D. DYCKMAN,² THOMAS W. HUBER,² AND ROBERT P. WILLIAMS¹

Houston City Health Department Laboratory² and Department of Microbiology and Immunology, Baylor College of Medicine,¹ Houston, Texas 77030

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We have shown that it is possible to screen for penicillinase-producing *Neisseria gonorrhoeae* directly from primary culture plates. Experiments involving the cocultivation of four genera of beta-lactamase-positive (β -lac⁺) bacteria and a beta-lactamase-negative (β -lac⁻) *N. gonorrhoeae* on modified Thayer-Martin medium indicated that suppressed or inhibited β -lac⁺ bacteria did not give false-positive results when isolated β -lac⁻ colonies of gonococci were tested. Colonies were assayed for β -lac production by an adaptation of a microacidometric method in which bacteria could be tested before or after the addition of oxidase reagent.

Penicillin is the drug of choice in the treatment of gonorrhea (3). Although strains of *Neisseria gonorrhoeae* that are relatively resistant to penicillin have been known for a number of years, the mechanism of this resistance was not thought to involve production of an enzyme capable of degrading the antibiotic (2, 8). Thus, penicillinase-producing *N. gonorrhoeae* (PPNG) isolated from infections contracted in the Far East and Liverpool, England, were unusual in that these organisms elaborated a beta-lactamase (β -lac) that conferred resistance to penicillin, ampicillin, and cephaloridine (1, 7).

As of 2 May 1977, 150 cases of disease caused by PPNG have been recognized in the United States (4). Determined efforts to locate and investigate the patients' sexual contacts apparently have successfully controlled the spread of these gonococci. However, because of the special threat posed by these bacteria, especially in terms of increased treatment costs, rapid and accurate susceptibility tests are important. Testing of growth from primary plates would save time, but current recommendations are to test reisolated organisms to prevent false-positive β -lac tests resulting from possible contaminants. Jorgensen et al. (5) recently reported a modification of an iodometric procedure for detecting β -lac-producing *N. gonorrhoeae* directly from colonies growing on the primary culture plate. Their procedure is more rapid than other tests currently available, since no further growth is required after the initial

isolation of the bacteria on Thayer-Martin medium. The possibility remains, however, that other β -lac-positive (β -lac⁺) bacteria present in the specimen taken for the diagnosis of gonorrhea could cause false-positive results.

In this communication, we analyze the feasibility of screening for PPNG from primary culture plates by examining the role of possible β -lac⁺ contaminants in causing spurious reactions. We also describe an adaptation of a microacidometric method (9) for the assay of β -lac production in which the suspect gonococcal colony is removed for testing either before or after the addition of oxidase reagent.

MATERIALS AND METHODS

Media. Modified Thayer-Martin medium (MTM) contained GC medium base (Difco), 1% hemoglobin (Difco), 1% defined supplement (IsoVitaleX; BBL), vancomycin (3.0 μ g/ml), colistin (7.5 μ g/ml), nystatin (12.5 U/ml), and trimethoprim lactate (5.0 μ g/ml). Chocolate agar contained the same constituents as MTM, except that the antimicrobial inhibitors were omitted. Heart infusion broth (Difco) was prepared according to the manufacturer's directions.

Test organisms. A β -lac-negative (β -lac⁻) strain of *N. gonorrhoeae* was obtained from the Texas Department of Health Resources in Austin. This organism exhibited a zone of inhibition measuring 36 mm by the penicillin disk diffusion test. Two β -lac⁺ strains were kindly made available by Clyde Thornsberry of the Center for Disease Control, Atlanta, Ga.; they were designated CDC3389 and CDC1782 by his laboratory. Both of these organisms showed no zone of inhibition by the penicillin disk diffusion test. Penicillinase-producing strains of

Escherichia coli and *Proteus rettgeri* were isolated from clinical specimens at the Hermann Hospital Microbiology Laboratory; β -lac⁺ strains of *Staphylococcus aureus* and *Flavobacterium meningosepticum* were isolated from clinical specimens at the Houston City Health Department Laboratory.

Preparation of β -lac test reagent and performance of the test. We used the microtiter modification of the acidometric test (9). If β -lac is present, penicillin is degraded to penicilloic acid that acidifies the medium and changes the color of the pH indicator. The indicator solution containing penicillin and phenol red was prepared as described previously (9). The test solution was either used immediately or dispensed in 2-ml samples in 1-dram (ca. 1.177 g) vials and frozen at -70°C . It was used as needed and could be refrozen and rethawed until it turned yellow.

The test was performed by dispensing 0.1 ml of penicillin-phenol red solution into wells of a microtiter plate (Cooke Engineering), one well for each culture to be tested. β -Lac⁺ and β -lac⁻ control organisms were included with each run. If the organism to be tested had been treated with oxidase reagent (*N,N*-dimethyl-*p*-phenylenediamine monohydrochloride; Eastman Kodak), control cultures likewise treated with oxidase reagent were included. Growth from one to five colonies of the bacteria was removed from the plates with a small bacteriological loop and suspended in the penicillin-phenol red solution. If the cultures produced β -lac, the solution usually turned yellow within 5 min; if the culture did not produce β -lac, the test solution remained dark red. The plates were allowed to stand for at least 30 min before a reaction was considered negative.

Determination of the feasibility of screening for PPNG from primary culture plates. A β -lac⁺ strain of *S. aureus*, *E. coli*, *F. meningosepticum*, or *P. rettgeri* and a β -lac⁻ strain of *N. gonorrhoeae* were suspended in heart infusion broth at a concentration equivalent to a standard McFarland I. The gonococci were further diluted 1:10, so that when mixed together, the β -lac⁺ bacteria were present at 10 times the concentration of the β -lac⁻ gonococci. These mixtures, as well as pure cultures of the test organisms, then were plated on MTM and chocolate agar, and the resultant growth was tested for β -lac reactivity.

RESULTS

Presumptive identification of *N. gonorrhoeae* at the Houston City Health Department Laboratory is made on the basis of colonial morphology and oxidase and Gram reactions. Since it is convenient to flood suspect colonies with oxidase reagent, we tested whether this reagent would interfere with the performance of the β -lac test (Table 1). A positive test was obtained with the β -lac⁺ *N. gonorrhoeae* both before and after exposure to oxidase reagent. Moreover, no false-positive results were obtained when β -lac⁻, oxidase-positive control cultures were tested.

TABLE 1. Effect of oxidase reagent on β -lac reactivity

Strain of <i>N. gonorrhoeae</i>	Exposure to oxidase reagent	
	Before addition	After addition
β -Lac ⁺	+	+ ^a
β -Lac ⁻	-	-

^a β -Lac reactivity was detected up to 9 h after oxidase was added to a 24-h culture; up to 5.5 h after oxidase was added to a 48-h culture; and up to 2.5 h after oxidase was added to a 72-h culture.

We next determined whether a false-positive β -lac test would result if β -lac⁻ gonococci were grown in the presence of a variety of β -lac⁺ bacteria. These organisms were mixed with the gonococcus as described in Materials and Methods. Either a loopful of bacteria was removed from the primary streak area (sweep) or an isolated colony (colony) was removed for testing (Table 2). In no case did exposure to oxidase reagent interfere with the performance of the test, although it did facilitate the identification of the gonococci, particularly in mixed cultures.

Whether plated on chocolate agar or MTM, the pure culture of β -lac⁻ *N. gonorrhoeae* gave a negative β -lac test. The pure cultures of *S. aureus*, *P. rettgeri*, *E. coli*, and *F. meningosepticum* gave positive tests from chocolate agar. The *S. aureus* and *E. coli* did not grow on MTM, so the β -lac test could not be done. Only one colony of *P. rettgeri* grew on MTM; this colony was β -lac⁺. Growth of the *F. meningosepticum* was greatly reduced on MTM, but colonies removed from this plate were β -lac⁺.

As expected, when the mixtures of organisms were plated, the *S. aureus*, *E. coli*, *F. meningosepticum*, and *P. rettgeri* overgrew the *N. gonorrhoeae* on chocolate agar. When a sweep was made of these plates, the β -lac test was invariably positive. However, when care was taken to pick only colonies resembling *N. gonorrhoeae*, a negative β -lac test was obtained. Small, gray, entire, mucoid colonies characteristic of *N. gonorrhoeae* could be picked without trouble, but the identification of gonococcal colonies was made particularly easy by the addition of oxidase reagent. When the mixtures of *S. aureus*, *E. coli*, or *P. rettgeri* and *N. gonorrhoeae* were plated on MTM, only colonies resembling *N. gonorrhoeae* were seen, and all the tests for β -lac were negative. There was a slight change in the color of the indicator solution when a sweep was made of the MTM plate containing *F. meningosepticum* and *N. gonorrhoeae*. This was attributed to the small number of gram-negative rods (i.e., β -lac⁺ *F.*

TABLE 2. Influence of a variety of penicillinase-producing bacteria on the β -lac reactivity of a penicillinase-negative *N. gonorrhoeae*

<i>N. gonorrhoeae</i> plus β -lac ⁺ inoculum ^a	β -Lac reaction							
	Chocolate agar				MTM			
	Without oxidase		With oxidase		Without oxidase		With oxidase	
	Sweep ^b	Colony ^c	Sweep	Colony	Sweep	Colony	Sweep	Colony
<i>S. aureus</i>	+	—	+	—	—	—	—	—
<i>F. meningosepticum</i>	+	—	+	—	Orange-yellow ^d	—	Orange-yellow	—
<i>E. coli</i>	+	—	+	—	—	—	—	—
<i>P. rettgeri</i>	+	—	+	—	—	—	—	—

^a The mixture was prepared so that the β -lac⁻ *N. gonorrhoeae* was present at one-tenth the concentration of the β -lac⁺ inoculum.

^b Sweep indicates that the inoculum for the test included a sample of all the growth on the plate.

^c One typical, well-isolated colony of *N. gonorrhoeae* was tested.

^d This well never turned frankly yellow; a Gram stain of the area on the plate from which the inoculum was removed showed a few gram-negative rods mixed with mostly gram-negative diplococci.

meningosepticum) seen on Gram stain of the inoculum. When isolated colonies of *N. gonorrhoeae* were removed from the plate for testing, a β -lac⁻ reaction was obtained. Thus, growth of β -lac⁻ *N. gonorrhoeae* in the presence of a suppressed β -lac⁺ bacterium did not result in a false-positive test for the gonococci.

DISCUSSION

The experiment involving cocultivation of β -lac⁺ bacteria and β -lac⁻ *N. gonorrhoeae* on MTM indicates that suppressed β -lac⁺ bacteria will not give false-positive results. Even in the case where the inoculum contained a few colonies of *F. meningosepticum* along with the *N. gonorrhoeae*, the indicator solution turned orange-yellow but never became yellow. Thus, the test would have been read as negative. However, we recommend that in instances where colonial morphology and Gram stain show mixed cultures, only well-isolated colonies be assayed for β -lac production. The addition of oxidase reagent to the plate simplifies the picking of colonies of gonococci for testing. Further, it is important to include known β -lac⁺ and β -lac⁻ control cultures with every run to ensure the proper function of the test.

The finding that β -lac activity can be assessed from primary culture plates will facilitate screening large populations for PPNG and quickly identifying patients in whom alternate therapy should be considered. During a 1-month period, for example, 1,641 primary cultures showing oxidase-positive colonies were screened at the Houston City Health Department Laboratory, using the microacidometric method described here. A presumptive identification of *N. gonorrhoeae* was made in 1,619 of the cultures; the remaining 22 cultures were

found to be gram-negative bacilli or coccobacilli. None of the gonococci tested displayed β -lac activity. These included isolates of *N. gonorrhoeae* from eight patients with positive cultures after routine penicillin administration, all of which exhibited zones of inhibition of ≥ 36 mm by the penicillin disk diffusion test. One of the nongonococcal cultures, subsequently identified as *F. meningosepticum*, gave a positive reaction.

We prefer the microacidometric method for β -lac detection to the paper strip modification of the iodometric procedure (5), because the endpoint is more easily read by the laboratorian, a large number of specimens can be processed with greater speed, and a smaller inoculum is required. During routine use, we have found that it is possible to test as little as one oxidase-positive colony of gram-negative diplococci, although the time required for the test to become positive is increased with smaller inocula. However, more than one isolated colony should be tested before calling a culture negative, because enzyme-negative colonies can represent 1 to 30% of the population obtained from primary clinical specimens of patients infected with β -lac⁺ *N. gonorrhoeae* (6).

We presently are using this procedure to test positive cultures of recently treated individuals who return for test of cure evaluation, and we plan to employ this protocol for routine screening of all *N. gonorrhoeae* isolates. In addition, we are using the test to examine the β -lac reactivity of other primary isolates, including *Haemophilus influenzae* and *Nocardia* sp.

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