Culture Medium for Confirmation of Penicillin-Resistant and Penicillinase-Producing Neisseria gonorrhoeae

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A culture method for the isolation and identification of penicillinase (β-lactamase)-producing Neisseria gonorrhoeae (PPNG) was evaluated in the Philippines where PPNG are common. The method uses plastic biplates containing standard Martin-Lewis gonococcal medium in one side of the biplate and PPNG-selective medium containing 1.5 μg of penicillin G per ml and a suspension of Sarcina lutea (Micrococcus lutea) that was susceptible to 0.01 μg of penicillin G per ml in the other side. Penicillin-resistant gonococci grow on both sides of the biplate. The hydrolysis of penicillin by β-lactamase permits the growth of S. lutea around PPNG colonies. With this medium we successfully identified 11 of 12 PPNG strains growing on primary isolation plates. A 48- to 72-h incubation period was needed, however, for visible growth of S. lutea around PPNG colonies. A unique advantage of this method was the identification of non-PPNG strains which also grew on penicillin-containing medium but did not allow growth of S. lutea. These relatively penicillin-resistant strains were the cause of infections which were not cured by penicillin treatment in 2 of 11 patients.

Penicillinase-producing Neisseria gonorrhoeae (PPNG) were first isolated in 1976 from patients who were not cured by treatment with recommended doses of aqueous procaine penicillin G (4). These organisms have since been isolated in at least 27 different countries and have achieved high prevalence in several areas of the Far East (5).

In the United States, a total of 2,419 cases of PPNG were reported to the Centers for Disease Control (CDC) from March 1976 through March 1981. The majority of the initial cases from which PPNG were isolated occurred in patients who became infected in the Far East before returning to the United States, or in their State-side contacts, but since July 1977 the majority of PPNG infections have not been linked to imported cases. A constant, low level of PPNG transmission may be responsible for endemic cases that are not imported (11). Because of the low prevalence of these strains, the CDC has recommended that tests for β-lactamase production be done only on N. gonorrhoeae isolated from patients whose infections have not responded to treatment with a recommended penicillin or ampicillin-probenecid regimen, on gonococci isolated from contacts of PPNG cases, or in areas where PPNG epidemics have occurred (6).

The accepted procedure for identifying PPNG is to isolate the organism in pure subculture and then to perform one of several different biochemical assays to confirm production of β-lactamase (11). Since the R-factor, or plasmid, which codes for the production of the β-lactamase may be lost when the organism is subcultured on penicillin-free media (12), and since a false-negative β-lactamase test may occur if less than 0.5% of the gonococci present in the culture are β-lactamase producers (β-lac+) (13), Martin and Lewis formulated a selective culture medium for primary isolation and confirmation of PPNG (8). Their medium selects penicillin-resistant organisms and differentiates between β-lactamase-producing (R-factor) and nonproducing (chromosomal) mechanisms for resistance.

In a laboratory evaluation of their PPNG-selective medium, Martin and Lewis identified 56 PPNG isolates out of a total of 111 gonococcal strains collected in the Philippines in September 1976 (8). Only 47 (84%) of these PPNG isolates were initially positive for β-lactamase production when tested by the chromogenic cephalosporin method or by the rapid iodometric method.

In an early field trial of the PPNG-selective medium in the Philippines, we found that both PPNG and non-PPNG could be isolated and

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identified on primary culture plates (P. L. Perine and J. W. Biddle, unpublished data). In this earlier study, the PPNG-selective medium did not contain vancomycin, and we found that staphylococci and other bacteria (some β-lac⁺) obscured the growth of N. gonorrhoeae or permitted growth of Sarcina lutea in cultures positive for β-lac⁺ N. gonorrhoeae. We report here the results of a second field trial of PPNG-selective medium which differed from the original formulation by the addition of vancomycin (4 μg/ml).

MATERIALS AND METHODS

Preparation of media. Penicillin selective medium was prepared at CDC. It contained the following ingredients dissolved in 960 ml of distilled water: GC base agar, 36.0 g; agar, 3.0 g; P.V.C.A.T. antibody solution, 10.0 ml; enrichment (IsoVitaleX), 10.0 ml; inactivated horse serum, 20.0 ml; and S. lutea suspension, 20.0 ml.

The agar (BBL Microbiology Systems, Cockeysville, Md.) was dissolved in the water, mixed, and autoclaved at 121°C for 15 min and then cooled to 48°C in a water bath before the other ingredients were added.

The P.V.C.A.T. antibiotic solution contained potassium benzyl penicillin, 0.15 mg (Sigma Chemical Co., St. Louis, Mo.); colistimethate sodium, 0.75 ml (Warner Chilcott, Morris Plains, N.J.); anisomycin, 0.20 ml (Pfizer Inc., New York, N.Y.); and trimethoprim lactate, 0.50 μg/ml (Burroughs Wellcome Co., Research Triangle Park, N.C.).

The horse serum (KC Biologicals, Lenexa, Kans.) was inactivated by heating at 56°C for 1 h.

The Martin-Lewis (ML) medium was prepared as previously described (9). The PPNG-selective medium was poured into one side of a plastic biplate (100 by 15 mm; Becton, Dickinson Labware, Oxnard, Calif.) and the ML medium was poured into the other. Prepared culture plates were sealed in plastic bags and stored at 4°C until used. The shelf-life of prepared and packaged PPNG medium stored at 2 to 8°C was 6 to 8 weeks.

Packaged culture plates were air-shipped to the Philippines in a refrigerated container and were warmed to ambient temperature before use. Referenced strains of N. gonorrhoeae with reproducible penicillin minimum inhibitory concentrations (MICs) indicating low (CDC F29; MIC, 0.015 μg/ml) and moderate (CDC F18; MIC, 0.5 μg/ml) chromosomally mediated resistance to penicillin, as well as a reference strain of PPNG (CDC 6-1782; MIC, 0.06 μg/ml) were used routinely as quality controls. There was no evidence of decreased penicillin activity or of a change in the quality of media used throughout the course of the study.

Preparation of vancomycin-resistant S. lutea suspension. A vancomycin-sensitive culture of S. lutea (FDA 1001) was inoculated onto selective chocolate agar (8) with a defined enrichment which contained increasing amounts of vancomycin to select mutants resistant to this antibiotic. The organism was subcultured daily on culture medium containing the same concentration of drug for 1 week, starting with medium containing 1.0 μg of vancomycin per ml. The vancomycin was increased by 1.0 μg/ml on the first day of each week until growth on medium containing 10.0 μg of vancomycin per ml was equivalent to growth in an inoculated control medium containing no inhibitory substances. A medium containing 10.0 μg of vancomycin per ml was used to grow the culture used for seeding. Aliquots of the seed culture were lyophilized and stored at 2 to 8°C until used. The seed suspension (no strain designation given) was prepared by washing the growth of a 24-h culture with Trypticase soy broth.

The resulting suspension was standardized by adding Trypticase soy broth to give a 1:100 dilution and then adding more of the broth to yield 40% light transmission in a Bausch & Lomb Spectronic 20 spectrophotometer at 530 nm. The penicillin MIC of the seed culture of S. lutea was not changed during selection of vancomycin-resistant mutants.

Collection of specimens. A group of 97 consecutive men and 133 consecutive women attending separate clinics in the same community in the Philippines were selected for culture. The men were volunteers, known sexual contacts of women with gonorrhea, or had symptoms of urethral discharge or dysuria or both. The women were asymptomatic and were undergoing routine screening cultures for gonorrhea. Urethral specimens were collected from men, and urethral or vaginal swabs were obtained from women by inserting a calcium alginate swab 1 to 2 cm into the anterior urethra; cotton swabs were used to obtain endocervical specimens in women. Each specimen was rolled over the surface of the agar on each side of the biplate in a random manner. Random inoculation of biplates carried the risk of transferring S. lutea to ML medium, but growth of S. lutea on ML medium was minimal and did not interfere with growth and identification of N. gonorrhoeae. Cultures were placed in a candle extinction jar immediately after inoculation and incubated at 36°C. Cultures were examined for growth after overnight incubation and then daily for 4 days.

Identification of gonococci and PPNG. Oxidase-positive, gram-negative diplococci with typical colony morphology were presumptively identified as N. gonorrhoeae and were immediately tested for β-lactamase by the chromogenic cephalosporin (Nirocefin) method (10). All presumptive gonococci were subcultured on chocolate agar. The susceptibility of pure cultures to penicillin was then determined by a disk agar diffusion method (2). The MIC of penicillin and the confirmatory sugar utilization tests for N. gonorrhoeae were determined for all isolates at the end of the study (14).

Treatment of patients. Men with urethritis who had a positive Gram stain for N. gonorrhoeae were immediately given 4.8 MU of aqueous procaine penicillin intramuscularly and 1.0 g of probenecid by mouth. If urethral cultures were positive for PPNG, the patient was recalled to the clinic and given spectinomycin, 2.0 g intramuscularly. This usually occurred within 48 h after initial treatment was given whether their urethral discharge had disappeared after penicillin treatment or not. Urethral cultures for N. gonorrhoeae were obtained from all male patients 5 to 7 days after treatment to confirm cure.

Women were given oral ampicillin, 3.5 g in a single dose, together with 1.0 g of probenecid if they had β-lac⁻ cultures, or spectinomycin 2.0 g intramuscularly if they were culture positive for PPNG. Women were
RESULTS

N. gonorrhoeae was isolated from the urethra and endocervix, respectively, of 26 of the 97 men (26.6%) and 10 of 133 women (7.5%) (Table 1). Of these, 8 of the 26 (31%) isolates from men and 4 of the 10 (40%) isolates from women were β-lac⁺ (Table 1). All of the isolates that grew on the PPNG medium also grew on ML medium.

Each of the three different assays for β-lactamase, i.e., chromogenic cephalosporin, disk test, and the penicillin selective medium, identified the eight PPNG isolates in men, but PPNG-selective media failed to identify β-lactamase production (no S. lutea growth) in one of the four PPNG strains isolated from women (Table 2). Although the chromogenic cephalosporin assay could be used to test for β-lactamase-producing gonococci that grew on ML medium after overnight incubation, macroscopic growth of S. lutea around β-lac⁺ colonies was not detected in PPNG-selective medium until after 44 to 68 h of incubation.

Only 12 of the 25 (48%) N. gonorrhoeae strains isolated on PPNG selective medium were β-lac⁺. The geometric mean concentration of the penicillin MIC for the 11 non-PPNG isolates which grew on PPNG-selective medium was significantly greater (t₁₅ = 3.19, P < 0.01) than the penicillin MIC of isolates that grew only on ML medium (Table 3).

Of the 26 men with culture-proven gonorrhea, 22 had positive Gram-stain smears of their urethral exudate and were treated with penicillin before the results of culture and β-lactamase tests were known. Only 9 of the 11 men infected with β-lac⁻ strains that grew on PPNG-selective medium and were treated with penicillin were cured. This relatively high treatment failure rate (18.2%) is probably related to the high penicillin MIC of the gonococcal strains (Table 3), since none of the men were sexually reexposed to infection after treatment. We were not able to determine the results of ampicillin treatment in the two women infected with non-PPNG which grew on penicillin selective media; both acknowledged sexual reexposure after treatment.

DISCUSSION

Blog et al. observed that PPNG may infect one site and non-PPNG may infect another in the same patient (3). Other investigators have found that β-lac⁺ and β-lac⁻ gonococcal colonies may coexist in as many as 42% of primary cultures (1). Thus, the isolation and identification of PPNG on primary culture depends on the site cultured and whether or not PPNG were present in the specimen collected for culture. Moreover, several investigators have found that the R-factor β-lactamase plasmid may be lost on the first or subsequent subcultures on penicillin-free medium (12, 13). These phenomena may explain why contacts of proven cases of PPNG may have only penicillin-susceptible gonococci isolated from infected sites (11). One of the potential advantages of PPNG-selective medium, which was not apparent in this study, would be the detection of small numbers of PPNG in primary culture that might otherwise be falsely negative by biochemical assays. We have consistently detected as few as five colony-forming units of N. gonorrhoeae in dilutions made of laboratory strains of β-lac⁺ N. gonorrhoeae when they were cultured on PPNG-selective medium.

If there is a reason (e.g., epidemiology) for determining the mechanism for penicillin resistance in as short a time as possible, then PPNG-selective medium may be preferred over one or more of the other methods used to detect β-lactamase production. The method used should be determined by the specific situation. If this information is not needed, the addition of S. lutea to penicillin-containing medium is a disadvantage.

A modification of PPNG-selective medium (ML medium containing penicillin but which does not contain S. lutea) has the potential to be very useful as a primary culture medium for detecting gonococcal infections in women residing in areas of the world which have a high prevalence of gonococci resistant to penicillin, whether this resistance is chromosomal or plasmid-mediated. All β-lac⁻ primary gonococcal isolates we recovered on PPNG-selective medium had an MIC of 0.5 μg/ml or greater. Patients...
infected with these strains should be treated with an antibiotic other than penicillin or ampicillin, since an unacceptable number of these patients will not be cured with these drugs (7).

The number of patients in this study was too small, however, to determine the usefulness of a penicillin-selective medium as a guide to therapy. Additional studies are in progress.

**ADDENDUM**

From March through June 1981, three sexually transmitted disease clinics in Los Angeles County, Calif., routinely used PPNG-selective medium for primary culture of patients suspected of having gonorrhea. Of 3,100 cultures done, 1,051 were positive for *N. gonorrhoeae*; 63 isolates grew on PPNG-selective medium, of which 46 were β-lac+ and the remainder were relatively penicillin-resistant non-PPNG strains. PPNG-selective medium identified five more PPNG-positive cultures than the conventional β-lactamase assay techniques in use (10 U penicillin disk or acido-metric test). The penicillin treatment failure rate in the 17 patients infected with relatively penicillin-resistant strains could not be determined, however, because all culture-positive patients were treated with spectinomycin in an attempt to prevent further increases in the prevalence of PPNG.

**LITERATURE CITED**