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

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Received 13 August 1981/Accepted 24 December 1981

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 gonorrhea culture 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
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-lact−) obscured the growth of N. gonorrhoeae or permitted growth of Sarcina lutea in cultures positive for β-lact− 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 (g), 36.0; agar (g), 3.0; P.V.C.A.T. antibiotic solution, 10.0 ml; enrichment (IsoViteX), 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 ml (Sigma Chemical Co., St. Louis, Mo.); colistin sulfate, 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; N. J.) 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 for culture from men 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-lact− cultures, or spectinomycin 2.0 g intramuscularly if they were culture positive for PPNG. Women were
grew

knowledge
two
determine
infection
(18.2%)
is
none
of
men
MIC
cured.
This
P-lac-
with
tests
and
medium
before
the
results
thral
exudate
and
had

grew
on
the
PPNG
strains
isolated
four
the
penicillin
incubation,
PPNG-selective
medium
until
after
the
PPNG
medium
test,
and
the
i.e.,
around
Iutea
gonococci
strains
isolated
The
of-cure
cultures
requested
although
to
say
endocervix,
selective
(2).

stub
2).

stub

Sex
No. cultured
No. of culture-positive N. gonorrhoeae
ML medium
PPNG medium
Male
97
26
19
Female
133
10
6

request
abstain
sexual
intercourse
3
to
7
days
after

RESULTS

N. gonorrhoeae
was
isolated
from
the
urethra
and
endocervix,
respectively,
of
26
of
the
97
men
(20.6%) 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-lacta-
mase,
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)
shorter
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
tested
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
to
determine
the
results
of
ampicillin

growth
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
coeexist
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
false
negative
by
biochemical
assays.
We
have
consistently
detected
as
few
as
five
colony-forming
units
of
N. gonorrhoeae
in
dilutions
made
of
labatory
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
disad-
vantage.

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


TABLE 3. Primary isolation of N. gonorrhoeae on PPNG medium as a predictor of the outcome of penicillin treatment in 26 men with gonorrhea

<table>
<thead>
<tr>
<th>Culture characteristics of the N. gonorrhoeae isolated</th>
<th>No. of patients</th>
<th>No. of patients not cured (%)</th>
<th>Distribution of penicillin by MICs (μg/ml)</th>
<th>Geometric mean MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-PPNG, grew only on ML medium</td>
<td>7</td>
<td>0</td>
<td>1 5 1</td>
<td>0.305</td>
</tr>
<tr>
<td>Non-PPNG, grew on ML and PPNG-selective medium</td>
<td>11</td>
<td>2 (18)</td>
<td>0 6 5</td>
<td>0.828</td>
</tr>
<tr>
<td>PPNG, grew on ML and PPNG-selective medium</td>
<td>8</td>
<td>8 (100)</td>
<td>0 0 8</td>
<td>7.336</td>
</tr>
</tbody>
</table>