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Inhibition of *Neisseria gonorrhoeae* isolates by Martin-Lewis medium

Epidemiology, susceptibility profile, and plasmid analysis

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SUMMARY A study was undertaken on the premise that if an increase of 1 μg/ml in the concentration of vancomycin in Martin-Lewis medium (MLM) could improve inhibition of Gram-positive cocci, then a concomitant increase in the inhibition of gonococci could also occur. Isolates of *Neisseria gonorrhoeae* that failed to grow on MLM accounted for 18·5% of all positive culture results for gonorrhoea. The incidence of isolates susceptible to vancomycin was 14% and of those susceptible to trimethoprim 2·4%; one isolate was susceptible to both vancomycin and trimethoprim. The antibiotic-susceptible isolates were more frequently isolated from asymptomatic white men. Plasmid analysis showed that the 2·4-megadalton cryptic plasmid was absent in the vancomycin-susceptible isolates. The large proportion of isolates failing to grow on selective MLM has important clinical implications for the diagnosis of gonorrhoea.

Introduction

The importance of cultural methods for the diagnosis of gonorrhoea is well documented and numerous modifications directed towards improving cultural yields have been published.[1] A modification of the Thayer-Martin medium[2] was introduced by Martin and Lewis in 1977,[3] in which the vancomycin concentration was increased from 3 μg/ml to 4 μg/ml, and the antifungal agent, nystatin, was replaced by anisomycin. The basis for this modification was to minimise overgrowth of cultures by yeasts and Gram-positive organisms such as staphylococci.

The new Martin-Lewis medium became the medium of choice in many sexually transmitted disease (STD) clinics and other laboratories processing cultures for gonorrhoea before it was subjected to sufficient clinical trials.[4] This caused concern because of the increase in concentration of vancomycin and reports of vancomycin-susceptible strains of *Neisseria gonorrhoeae*.[5-10]

This paper reports on the inhibitory qualities of the Martin-Lewis formulation, the susceptibility profiles of isolates failing to grow on this medium, and a plasmid analysis of these isolates.

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Materials and methods

CULTURAL PROCEDURES

The specimens were obtained from consecutive outpatients examined at the STD clinic, Peoria City/County Health Department, Peoria, Illinois, between 12 February and 22 March 1979. Specimens were collected from the urethra of men and the cervix of women. Men with a history or evidence of urethral discharge or dysuria or both were classed as symptomatic. Patients with no history or evidence of discharge and dysuria were classed as asymptomatic.

Martin-Lewis medium (MLM) containing 4 μg/ml vancomycin, 7·5 μg/ml colistin, 20 μg/ml anisomycin, and 5 μg/ml trimethoprim lactate was supplied in a single lot by Scott Laboratories, Fiskeville, RI, USA. Quality control was performed by the manufacturer. Chocolate agar plates (CAP) with 1% haemoglobin and 1% supplement C (Difco, Detroit, Michigan) were made daily.

Specimen swabs were immediately placed into a labelled tube of 5·0 ml brain heart infusion broth (BHI, Difco, Detroit) and processed in the laboratory within 15-30 minutes of receipt. The swab and broth

were vortexed for 20 seconds and the swab removed. Fresh sterile swabs (Dacron, No A5005-1, American Scientific Products) were immersed in the BHI for 20 seconds and an 'N' configuration streaked on to MLM and CAP; a fresh swab was used to streak each medium. The 'N' configuration was cross-streaked with a sterile loop and incubated at 35°C in 6% CO_2 with readings at 48 and 72 hours. Gram-negative oxidase-positive diplococci were identified by a carbohydrate utilisation test using the medium described by Flynn and Waitkins,[11] with the addition of 3·5 μg/ml cocarboxylase (Sigma, St Louis, Missouri). Heat-fixed suspensions of vancomycin-susceptible isolates were also treated with antigonococcal globulin conjugated with fluorescein isothiocyanate for identification purposes.

SUSCEPTIBILITY TESTING

Minimum inhibitory concentrations (MIC) were determined in Mueller-Hinton broth with 1% supplement C (Difco, Detroit) and by an agar dilution method using GC agar base with 1% supplement C and 1% haemoglobin.[12] An inoculum of 5×10^5 organisms was used. Susceptibility testing for trimethoprim was performed in trypticase soy media (Difco, Detroit) supplemented with 1% Isovitalex. Cultures were incubated at 35°C in 6% CO_2 and read after 24, 48, and 72 hours.

Vancomycin hydrochloride (Vancocin® HCl) was supplied as a sterile dry powder in 500-mg vials (Eli Lilly and Co, Indianapolis). Trimethoprim lactate (Burroughs Wellcome Co, Research Triangle Park, North Carolina), spectinomycin sulphate (Trobicin, Upjohn Company, Kalamazoo, Michigan), cefoxitin (Merck, Sharp and Dohme, Rahway, NJ), and commercial preparations of penicillin G and tetracycline hydrochloride (Pfizer Inc, New York) were used.

CULTURE AND DNA EXTRACTION

N gonorrhoeae isolates to be examined for plasmids were grown in 500 ml trypticase soy broth (TSB) containing 0·01 mol/l $MgSO_4$ and 0·01 mol/l $NaHCO_3$, pH 7·2, for 8-12 hours at 35°C. *E coli* strain V517, which contained plasmids of known specific molecular weights, was supplied by Dr Ester M Lederberg (Plasmid Reference Center, Stanford University School of Medicine, Stanford, California). Cultures for extraction purposes were grown in TSB and harvested after 12 hours' incubation at 35°C. The DNA extraction procedure included the Guerry *et al* [13] method for lysing cells followed by the ribonuclease, deproteinisation, and precipitation steps described by Meyers *et al.*[14]

AGAROSE GEL ELECTROPHORESIS

The 0·7% or 1·0% agarose (w/v) gel was electrophoresed at 30 mA at room temperature until the dye was 10 mm from the bottom of the gel. The gel was then placed in a solution of ethidium bromide (0·05 μg/ml) and stained for 15 min.[15,16] Gels were photographed on a black background under ultraviolet light (375 nm) for 15-20 min using a 35 mm camera with Wratten No 22 orange filter.

STATISTICAL ANALYSIS

Results were evaluated for statistical significance by Student's *t* test.

Results

CLINICAL AND CULTURE RESULTS

A total of 346 men were examined and gonococci isolated from 58 (16·8%); 105 women were examined and gonococci isolated from 23 (21·4%). Of the positive cultures, 10 of 58 (17·2%) from men and five of 23 (21·7%) from women failed to grow on MLM after 96 hours of incubation at 35°C in 6% CO_2. During repetitive subculturing, two isolates were lost from a white symptomatic man and a white asymptomatic woman. Therefore, 13 of 79 (16·5%) positive cultures that failed to grow on MLM remained for further testing.

Details of the patients from whom these 13 strains were isolated are shown in table I. The proportion of isolates failing to grow on selective MLM in men (17·2%) and women (21·7%) was comparable. The frequency of recovery of such isolates was three times greater in white than in non-white patients and the individual was more likely (2½ times) to be an asymptomatic white man.

TABLE I *Clinical features of 13 patients infected with gonococci which failed to grow on selective (MLM) media.*

	Race			
	White		*Non-white**	
Sex	*Sympto-matic*	*Asympto-matic*	*Sympto-matic*	*Asympto-matic*
Male	2	5	2	0
Female	2	1	0	1

* All were black

SUSCEPTIBILITY PROFILES

Vancomycin MICs of isolates failing to grow on the selective MLM media were <2 μg/ml for nine isolates, >2 but <3 μg/ml for two, and >8 μg/ml for the remaining two strains. The rate of vancomycin susceptibility (MIC <3 μg/ml) was 14% of all positive cultures for *N gonorrhoeae*. One isolate which failed to

grow on the selective medium was resistant to vancomycin but susceptible to trimethoprim. Another isolate was inhibited by both vancomycin and trimethoprim. Susceptibility to trimethoprim was 2·4% of the total positive cultures.

All of the 13 isolates failing to grow on MLM were susceptible to <1 μg/ml penicillin (range 0·011-0·78 μg/ml). Susceptibility was noted to tetracycline (range 0·011-1·56 μg/ml), spectinomycin (range 0·045-12·5 μg/ml), and cefoxitin (range 0·045-1·56 μg/ml). Thirteen control isolates, matched for the patients' age, sex, race, and day and week of collection were examined. The mean MIC for each antibiotic for the vancomycin-sensitive and control organisms is shown in table II. The data between the groups were compared by a standard Student *t* test. There was a significant difference ($P > 0.997$) in the mean vancomycin MIC between the two groups of isolates but no significant difference in the mean MICs for the other antibiotics.

With a high inoculum (10^6 cfu) four of 13 isolates, originally failing to grow on MLM, overcame the inhibition of vancomycin on subculture. Only two of the four did so, however, when a 10^3 inoculum was used. The strains which grew on vancomycin-containing plates with both inocula had MICs of 12·5 and 25 μg/ml. The other two isolates had MICs of 2·5 μg/ml. All isolates with MICs <2·0 μg/ml failed to grow on vancomycin-containing media regardless of the inoculum size. Neither of the two trimethoprim-sensitive isolates grew when subcultured to trimethoprim-containing media with inocula of either 10^3 or 10^6 cfu.

PLASMID ANALYSIS

Six isolates which failed to grow on MLM were analysed for their plasmid content and compared with six isolates with vancomycin MICs >8 μg/ml. The gel patterns of two isolates with vancomycin MICs >8 μg/ml are shown in the figure (lanes 2 and 3). Compared with standard molecular-weight plasmids in lane 1, a single 2·6-megadalton plasmid was observed in both isolates. Lane 4 contains an isolate with a vancomycin MIC of 2·5 μg/ml which overcame the inhibition of vancomycin only with an inoculum of 10^6 cfu. A 2·6-megadalton plasmid is evident.

Lanes 5-7 show three vancomycin-susceptible isolates, with lane 7 representing the isolate susceptible to both vancomycin and trimethoprim. The 2·6-megadalton plasmid is absent in each of these three isolates. In preparations (not shown) of two additional isolates susceptible to vancomycin, the 2·6 megadalton plasmid was also absent. All control isolates tested (vancomycin MIC >8 μg/ml) contained the 2·6-megadalton plasmid as did the two isolates which initially failed to grow on MLM but were later found to be vancomycin-resistant. No 25-megadalton plasmid was observed in any of the isolates studied.

Discussion

Isolates of *N gonorrhoeae* that failed to grow on MLM accounted for 18·5% of organisms isolated over a six-week period. Of the 15 isolates recovered, 13 were further evaluated; 11 (14%) were susceptible to <3 μg/ml vancomycin. Susceptibility to trimethoprim was observed in two (2.4%) isolates. While approximately 10 reports on vancomycin susceptibility have been published since 1969, fewer have cited susceptibility to trimethoprim.[5,17-19] Taylor and Phillips[5] reported that only 1% of their isolates had MICs <4 μg/ml. Our data illustrate a similar small proportion of isolates inhibited by concentrations of trimethoprim present in MLM. This would indicate that trimethoprim should not present any undue problem of inhibition of strains when incorporated into selective media.

In other studies the incidence of susceptibility to vancomycin has been around 3-4%[5-7] and 8-12%.[8-10] The 14% incidence of vancomycin susceptibility in this study falls in the latter group. Windall *et al* have reported a 30% incidence of gonococcal cultures failing to grow on modified Thayer-Martin media.[20]

There is little published information on the relationship between vancomycin-susceptible strains and MICs to other antibiotics. Antibiotic susceptibility is a mark of virulence for some gonococci.[21,22] Marked susceptibility to penicillin (MIC ≤0·015 μg/ml) has been found in strains from disseminated gonococcal infection (DGI)[21,23] and from patients with

TABLE II *Mean MICs of six antibiotics for test and control organisms*

	Mean MIC (μg/ml) ± SD of following antibiotics:					
*Organisms**	*VAN*	*TMP*	*PEN*	*TET*	*SPC*	*CFX*
Test	3·43 ± 7·31	71·42 ± 40·12	0·46 ± 0·55	0·76 ± 0·65	5·23 ± 5·17	0·37 ± 0·39
Control	30·28 ± 26·25	61·53 ± 33·25	0·42 ± 0·31	0·76 ± 0·49	6·55 ± 5·09	0·36 ± 0·38

VAN = vancomycin; TMP = trimethoprim; PEN = penicillin; TET = tetracycline; SPC = spectinomycin; CFX = cefoxitin; SD = standard deviation.

* Three MIC values were estimated for each of 13 isolates in both test and control groups.

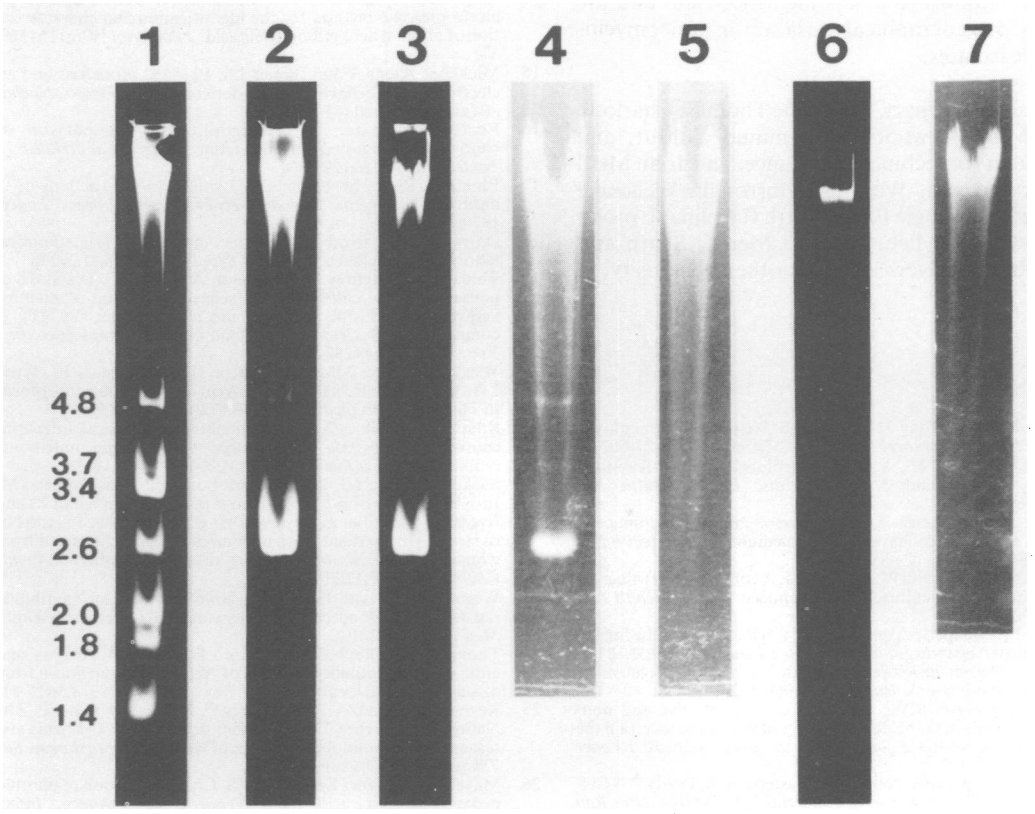

FIGURE *Agarose gel electrophoresis of plasmid DNA. Lane 1 contains E coli strain V517 with standard molecular-weight plasmids. Lanes 2 and 3 show vancomycin-resistant isolates (MIC > 4 μg/ml), which contain a 2·6-megadalton plasmid. Lane 4 contains a vancomycin-susceptible isolate that was able to grow in the presence of vancomycin with a large (10^6 cfu) inoculum and had a vancomycin MIC of 2·5 μg/ml. Isolates with vancomycin MICs <2·0 μg/ml (lanes 5-7) did not contain a 2·6-megadalton plasmid.*

asymptomatic urethral infections. The penicillin MICs for our vancomycin-susceptible isolates were comparable to those of Thompson *et al*[24] for non-DGI isolates, with 72% of all isolates having penicillin MICs ≥0·125 μg/ml. Our findings for susceptibility to tetracycline were, however, similar to those for DGI isolates[24] with 60% having MICs of 0·125-0·250 μg/ml. These MICs of tetracycline are equivalent to those reported for gonococcal isolates examined in the National Gonorrhea Therapy Monitoring Study (NTMS)[25] from 1972 to 1977. MICs for spectinomycin and cefoxitin were comparable to NTMS results.[25]

Many drug resistant markers in bacteria are located on plasmids. Agarose gel electrophoresis of vancomycin-susceptible isolates showed the absence of the indigenous 2·6-megadalton plasmid when the vancomycin MIC was <2·0 μg/ml. While this plasmid does not universally appear in gonococcal isolates, reported rates range between 83-100%.[26-28] Sparling *et al*[29] have attributed vancomycin susceptibility to envelope (*env*) mutations. Although the outer membrane of these isolates has not been analysed, recent data from Miller *et al*[12] suggest that the cytoplasmic membrane may also be an important determinant of antibiotic susceptibility or resistance in *N gonorrhoeae*. While the loci for *env* mutations are purported to be chromosomally located,[30] further genetic analysis of these vancomycin-susceptible isolates are warranted in view of our plasmid findings.

The large proportion of isolates failing to grow on selective MLM has clinical implications for the diagnosis of gonorrhoea. This report is to alert both physicians and laboratory personnel to yet another basis for false-negative culture results. A change in antibiotic composition of gonococcal selective media may be warranted. Additional studies are in progress

throughout Illinois to assess incidence rates and the possibility of geographical variation in vancomycin-susceptible isolates.

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References

1. Martin JE, Armstrong JH, Smith PB. New system for cultivation of *Neisseria gonorrhoeae*. *Appl Microbiol* 1974;27:802-5.
2. Thayer JD, Martin JE. A selective medium for the cultivation of *N gonorrhoeae* and *N meningitidis*. *Public Health Rep* 1964;79:49-57.
3. Martin JE jun, Lewis J. Anisomycin: improved antimicotic activity in modified Thayer-Martin medium. *Public Health Lab* 1977;35:53-60.
4. Smeltzer MP, Curran JW, Lossick JG. A comparative evaluation of media used to culture *N gonorrhoeae*. *Public Health Lab* 1979;37:43-56.
5. Taylor E, Phillips I. Assessment of a selective medium for the isolation of *Neisseria gonorrhoeae*. *Br J Vener Dis* 1979;55:183-5.
6. Reyn A. Recent developments in the laboratory diagnosis of gonococcal infections. *Bull WHO* 1969;40:245-55.
7. Reyn A, Bentzon MW. Comparison of a selective and non-selective medium in the diagnosis of gonorrhoea to ascertain the sensitivity of *Neisseria gonorrhoeae* to vancomycin. *Br J Vener Dis* 1972;48:363-8.
8. Cross RC, Hoger MB, Neibaur R, Pasterneck B, Brady F. VCN-inhibited strains of *Neisseria gonorrhoeae*. *HSMHA Hlth Rep* 1971;86:990-2.
9. Brorson JE, Holmberg I, Nygren B, Seaberg S. Vancomycin-sensitive strains of *Neisseria gonorrhoeae*. A problem for the diagnostic laboratory. *Br J Vener Dis* 1973;49:452-3.
10. Phillips I, Humphrey D, Middleton A, Nicol CS. Diagnosis of gonorrhoea by culture on a selective medium containing vancomycin, colistin, nystatin, and trimethoprim (VCNT). A comparison with Gram staining and immunofluorescence. *Br J Vener Dis* 1972;48:287-92.
11. Flynn J, Waitkins SA. A serum-free medium for testing fermentation reactions in *Neisseria gonorrhoeae*. *J Clin Pathol* 1972;25:525-7.
12. Miller MA, Scharli CS, Mills J. Cytoplasmic membrane proteins of spectinomycin-susceptible and -resistant strains of *Neisseria gonorrhoeae*. *J Bacteriol* 1980;143:198-204.
13. Guerry P, LeBlanc D, Falkow S. General method for the isolation of plasmid deoxyribonucleic acid. *J Bacteriol* 1973;116:1064-6.
14. Meyers J, Sanchez D, Elwell L, Falkow S. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *J Bacteriol* 1976;127:1529-37.
15. Mickel S, Arena V jun, Bauer LS. Physical properties and gel electrophoresis behavior of RIZ-derived plasmid DNAs. *Nucleic Acids Res* 1977;4:1465-82.
16. Foster R, Foster G. Electrophoretic comparison of endonuclease-digested plasmids from *Neisseria gonorrhoeae*. *J Bacteriol* 1976;126:1297-304.
17. Phillips I, Ridley M, Rimmer D, Lynn R. In-vitro activity of 12 antibacterial agents against *Neisseria gonorrhoeae*. *Lancet* 1970;i:263-5.
18. Darrell JH, Garrod LP, Waterworth PM. Trimethoprim: laboratory and clinical studies. *J Clin Pathol* 1968;21:202-9.
19. Phillips I, Humphrey D, Middleton A, Nicol CS. Diagnosis of gonorrhoea by culture on a selective medium containing vancomycin, colistin, nystatin, and trimethoprim (VCNT): A comparison with Gram-staining and immunofluorescence. *Br J Vener Dis* 1972;48:287-92.
20. Windall JJ, Hace MM, Washington II JA, Douglass TJ, Weed LA. Inhibitory effects of vancomycin on *Neisseria gonorrhoeae* in Thayer-Martin medium. *J Infect Dis* 1980;142:775.
21. Knapp JS, Holmes KK. Disseminated gonococcal infections caused by *Neisseria gonorrhoeae* with unique nutritional requirements. *J Infect Dis* 1975;132:204-8.
22. Sackel SG, Alpert S, Rosner B, McLormack WM, Findland M. In-vitro activity of ρ-hydroxybenzyl penicillin (penicillin X) and five other penicillins against *Nesseria gonorrhoeae*: comparisons of strains from patients with uncomplicated infections and from women with pelvic inflammatory disease. *Antimicrob Agents Chemother* 1977;12:31-6.
23. Weisner PJ, Handsfield HH, Holmes KK. Low antibiotic resistance of gonococci causing disseminated infection. *N Engl J Med* 1973;288:1221-2.
24. Thompson S, Reynolds G, Short H, *et al.* Auxotypes and antibiotic susceptibility patterns of *Neisseria gonorrhoeae* from disseminated and local infections. *Sex Transm Dis* 1978;5:127-31.
25. Reynolds G, Ziadi A, Thornsberry C, Biddle J, Wiesner P. The National Gonorrhea Therapy Monitoring Study: II Trends and seasonality of antibiotic resistance of *Neisseria gonorrhoeae*. *Sex Transm Dis* 1979;6 suppl:103-11.
26. Mayer LW, Holmes KK, Falkow S. Characterization of plasmid deoxyribonucleic acid from *Neisseria gonorrhoeae*. *Infect Immun* 1974;10:712-7.
27. Elwell LP, Falkow S. Plasmids of the genus *Neisseria*. In: *The Gonococcus*, Roberts RB, ed. New York: John Wiley & Sons, 1977;138-54.
28. Roberts M, Elwell L, Falkow S. Introduction to the mechanisms of genetic exchange in the gonococcus: Plasmids and conjugation in *Neisseria gonorrhoeae*. In: Brooks G, Gotschlich E, Holmes K, Sawyer W, Young F eds. *Immunobiology of Neisseria gonorrhoeae*. Washington DC: American Society for Microbiology, 1978;38-43.
29. Sparling PF, Guymon L, Biswas G. Antibiotic resistance in the gonococcus. In: Schlessinger D ed. *Microbiology – 1976*. Washington DC: American Society for Microbiology, 1976;44-52.
30. Maness M, Sparling PF. Multiple antibiotic resistances due to a single mutation in *Neisseria gonorrhoeae*. *J Infect Dis* 1973;128:321-30.