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Development of an Antimicrobial Susceptibility Surveillance System for *Neisseria gonorrhoeae* in Malawi: Comparison of Methods

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Susceptibility of *Neisseria gonorrhoeae* to gentamicin, the primary treatment for gonorrhea in Malawi since 1993, was determined by using agar dilution MICs, E-test MICs, disc diffusion, and clinical cure rate. Agar dilution MICs were slightly higher in 1996 than in 1993 isolates, with a concomitant drop in the clinical cure rate. E-test MICs were substantially lower than agar dilution determinations, with only 77.4% within 1 $\log_2$ concentration.

Rates of gonorrhea infection are high worldwide, especially in developing countries (21). In Malawi, cervical infection with *Neisseria gonorrhoeae* was found in 5% of urban antenatal women, comparable to infection rates in low-risk populations in other countries in the region (6, 9). Gonococcal infections facilitate the transmission of human immunodeficiency virus (10), and increased shedding of human immunodeficiency virus type 1 in semen of infected men has been demonstrated (3).

In Malawi, penicillin was the drug of choice for the treatment of gonorrhea until clinical efficacy studies and agar dilution MIC determinations carried out by the AIDS Control and Prevention Project from 1991 to 1993 found high-level gonococcal resistance to penicillin, tetracycline, and streptomycin (12). Based on clinical efficacy, MIC data, and cost, gentamicin (240-mg single intramuscular dose) was adopted in 1993 as the treatment of choice for gonorrhea in Malawi (13, 14). Single-dose gentamicin has been shown to be effective in the treatment of gonococcal urethritis and cervical infection (2, 7, 8, 15, 17, 19). Since early 1994, gentamicin has been widely used in the syndromic treatment of all urethral discharge but no studies of its continued efficacy have been performed.

Monitoring of the antimicrobial susceptibility of *N. gonorrhoeae* in developing countries is essential in an environment of rapidly changing resistance patterns (5, 11). However, surveillance systems involving clinical efficacy studies and agar dilution methods for determining MICs are time-consuming and expensive. In addition, the laboratory capability for agar dilution testing is limited to reference laboratories in developed countries. While these methods may be feasible for donor-funded special studies, they are not sustainable for long-term monitoring.

The objectives of the present study were to determine the clinical efficacy of gentamicin in the treatment of *N. gonorrhoeae* 2 years after the onset of its widespread use and to evaluate the possible roles of disc diffusion and the E test in the development of a surveillance system in Malawi for monitoring the susceptibility of *N. gonorrhoeae* to gentamicin.

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Study population. This study was conducted at Lilongwe Central Hospital in Lilongwe, Malawi, between January and March 1996. Also presented are data from 198 male patients at Queen Elizabeth Central Hospital, Blantyre, Malawi, between September 1992 and March 1993 as part of a larger study to determine effective urethritis therapy (13).

Clinical specimens. After administration of a questionnaire and a physical examination, urethral swab specimens were obtained for microscopy of the Gram-stained smear and for culture of *N. gonorrhoeae*. After giving informed consent, 206 consecutive men presenting to the sexually transmitted disease clinic in Lilongwe with complaints of urethral discharge were enrolled. Gonorrhea was presumptively diagnosed by the presence of gram-negative intracellular diplococci (GNID) on the first visit and confirmed by culture. Subjects were asked to return in 7 days, and a follow-up urethral swab specimen was taken for repeat Gram stain and culture. A stipend was given for return visits.

Urethral specimens were directly inoculated onto chocolate agar (GC agar base, bovine hemoglobin, IsoVitaleX) and immediately placed in candle extinction jars. Cultures were incubated at 35°C and inspected for growth indicative of *N. gonorrhoeae* after 24 and 48 h. A Gram stain was performed on oxidase-positive colonies with characteristic morphology. Isolates were subcultured for 24 h on chocolate agar and then used for E-test and disc diffusion analysis. Each isolate was placed in transport medium and stored in liquid nitrogen until shipment on dry ice to the University of North Carolina for MIC determination by the agar dilution method.

All patients presenting with urethral discharge had urethritis, defined as the presence of five or more leukocytes per high-power field on Gram stain, and 51.9% (107 of 206) had cultures positive for *N. gonorrhoeae*. The mean reported duration of urethral discharge was 14 days (median, 7 days; range, 0 to 360 days). Although 51.5% reported treatment with an antibiotic in the previous 2 weeks, only one subject had received treatment effective against gonorrhea (gentamicin at an unknown dosage).

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Treatment and clinical efficacy of gentamicin. At the initial visit, men with urethritis and GNID on Gram stains were treated with gentamicin (240-mg single intramuscular dose). Failure of treatment for gonorrhea was defined as persistent GNID on Gram stain after 1 week or a positive *N. gonorrhoeae* culture. Men with persistent urethritis after 1 week were treated with ciprofloxacin (500-mg single oral dose) and azithromycin (1-g single oral dose).

Of the 107 patients with a positive gonorrhea culture on the first visit, 90 (84%) returned for a follow-up culture. Of the returning patients, five were excluded from the analysis of clinical efficacy: four received azithromycin on the first treatment visit in addition to gentamicin, and for one with a positive gonococcal culture on the second visit, the gentamicin MIC was substantially lower than on the initial visit (8 versus 32 μg/ml) and was considered to be reinfected, despite denying having had sexual intercourse between cultures, as did all subjects with persistent gonorrhea after treatment. The clinical cure rate for gentamicin treatment of gonorrhea was 91.8% ($n = 85$; 95% confidence interval, 83.8 to 96.6%) in 1996 compared to 95% ($n = 40$; 95% confidence interval, 83.8 to 99.4%) in 1993 (13). Although the treatment failure rate was slightly increased in 1996, the difference was not statistically significant.

Microbial susceptibility testing. We compared disc diffusion and E-test gentamicin susceptibility determinations for *N. gonorrhoeae* with the "gold standard" for MICs, the agar dilution technique. Both disc diffusion and the E test are widely available, low-technology methods requiring only the ability to carry out *N. gonorrhoeae* cultures on agar plates. The E test has proven useful in measuring gonococcal susceptibility to a variety of antibiotics (4, 18, 20, 22); however, none of these studies was performed in a developing country field laboratory setting.

For disc diffusion and E-test susceptibility determinations, inocula were prepared with *N. gonorrhoeae* isolates suspended in saline to a turbidity equivalent by inspection with the naked eye to a 0.5 McFarland standard. Chocolate agar plates were streaked over the entire surface with a wire loop to deliver the bacterial suspension. A 10-μg gentamicin disc (BBL Sensi Disc Antimicrobial Susceptibility test discs; Becton Dickinson) and a gentamicin E-test strip (AB Biodisk North America, Inc., Piscataway, N.J.) containing an exponential antibiotic gradient on one side and an MIC reading scale marked on the other were firmly placed on the plate. Plates were incubated for 24 h at 35°C in candle extinction jars.

The diameter of the growth inhibition zone surrounding the gentamicin disc was read to the nearest millimeter. The diameters of *N. gonorrhoeae* inhibition zones by disc diffusion ranged from 15 to 22 mm (data not shown). There were no significant differences in the diameters of inhibition zones at different MICs by agar dilution (Pearson's chi square, 17.0; $P = 0.95$).

E-test MICs were determined by reading the value at the point of intersection between the edge of the zone of growth inhibition and the E-test strip. Intermediate E-test values falling between standard agar dilutions were rounded up to the next highest twofold dilution, as suggested by the manufacturer. An *Escherichia coli* control strain (ATCC 25922) with a standard gentamicin E-test range of 0.25 to 1.00 μg/ml and a 10-μg disc diameter of 19 to 26 mm was used as a control before and then monthly during the 3-month trial. Disc diffusion and E-test values for the *E. coli* control strain consistently fell within the standard ranges.

MIC determination by agar dilution followed the current Centers for Disease Control and Prevention guidelines for the National Gonococcal Isolate Surveillance Project (U.S. Public Health Service, Center for Infectious Diseases, Division of Sexually Transmitted Diseases Laboratory Research, Atlanta, Ga.). Susceptibilities were determined on GC agar base medium supplemented with 1% IsoVitaleX inoculated with 10^4 CFU of each strain. Gentamicin and other antibiotics were serially diluted into a series of plates for concentrations of 0.001, 0.015, 0.06, and 0.12 μg/ml and doubling concentrations from 0.5 to 256 μg/ml. The agar dilution MIC was defined as the lowest concentration of antibiotic that completely inhibited growth of the inoculum. MICs of penicillin and tetracycline for *N. gonorrhoeae* showed that >83% of the isolates were resistant to these antibiotics (data not shown). Isolates were uniformly sensitive (MICs, <0.125 μg/ml) to ciprofloxacin ($n = 44$), cefixime ($n = 63$), and ofloxacin ($n = 63$).

TABLE 1. Gentamicin susceptibility[a] of *N. gonorrhoeae* isolates from Malawi

Yr of isolation	No. of isolates for which agar dilution MIC (μg/ml) was:							No. of isolates
	0.5	1	2	4	8	16	32	
1993	2[b]	1[b]	2[b]	29[b]	119[c]	44[c]	1[d]	198
1996			2[e]	4[e]	32[f]	23[f]	2[g]	63

[a] Based on interpretative criteria for aerobic bacteria other than *S. pneumoniae*, *Haemophilus* spp., and *N. gonorrhoeae* (16).
[b] Percentage of high-susceptibility isolates, 17.2%.
[c] Percentage of moderate-susceptibility isolates, 82.3%.
[d] Percentage of low-susceptibility isolates, 0.5%.
[e] Percentage of high-susceptibility isolates, 9.5%.
[f] Percentage of moderate-susceptibility isolates, 87.3%.
[g] Percentage of low-susceptibility isolates, 3.2%.

Agar dilution MICs of gentamicin are summarized in Table 1. The comparison of gentamicin MICs from 1993 and 1996 revealed a statistically significant difference by the Wilcoxon rank-sum test ($P < 0.01$), with the proportion of highly susceptible isolates falling from 17.2% in 1993 to 9.5% in 1996. However, the median MIC (8 μg/ml) was unchanged from 1993 to 1996 and the majority of isolates remained in the moderately susceptible category.

In 1993, 40 of the 198 isolates were treated with gentamicin. There were two treatment failures, for both of which the MIC was ≥16 μg/ml. Of those treated with gentamicin, the MICs for eight isolates were ≥16 μg/ml. Three were lost to follow-up; of the five returning, two were treatment failures. In 1996, the MICs for four of the seven patients experiencing treatment failure with a single dose of gentamicin ranged from 4 to 16 μg/ml (one was 4 μg/ml, two were 8 μg/ml, and one was 16 μg/ml).

There are no standardized interpretative criteria for gentamicin MICs in the treatment of *N. gonorrhoeae*. By the National Committee for Clinical Laboratory Standards criteria for aerobic bacteria other than *N. gonorrhoeae*, *Streptococcus pneumoniae*, and *Haemophilus* species (16), three of the four treatment failures in 1996 and both of the failures in 1993 were in the category of moderate susceptibility. It is interesting that of the two isolates for which the MIC was 32 μg/ml in 1996, one responded to gentamicin treatment and one patient failed gentamicin therapy but when he returned for a second visit, the MIC was 8 μg/ml, suggesting reinfection.

Comparison of MICs determined by agar dilution and the E test. Table 2 shows gentamicin MICs for *N. gonorrhoeae* isolates determined by agar dilution assay and the E test. Consistent with other reports, the E test gave lower MICs than did the agar dilution assay. Other studies have reported agree-

TABLE 2. Comparison of agar dilution assay and E-test results for *N. gonorrhoeae* susceptibility to gentamicin in Malawi in 1996[a]

Method	No. of isolates for which MIC (μg/ml) was:							Total no. of isolates
	0.5	1	2	4	8	16	32	
E test			4[b]	60[b]	43[c]	0[c]	0	107
Agar dilution			2[d]	4[d]	32[e]	23[e]	2[f]	63

[a] Based on interpretative criteria for aerobic bacteria other than *S. pneumoniae*, *Haemophilus* spp., and *N. gonorrhoeae* (16).
[b] Percentage of high-susceptibility isolates, 60.4%.
[c] Percentage of moderate-susceptibility isolates, 39.6%.
[d] Percentage of high-susceptibility isolates, 9.5%.
[e] Percentage of moderate-susceptibility isolates, 87.3%.
[f] Percentage of low-susceptibility isolates, 3.2%.

ments of 76 to 98%, depending on the study and on the antibiotic used (1, 4, 18, 20, 22). In studies reporting the lowest agreement (4, 20), there were no differences in the susceptibility category of the isolates between methods. We found a large discrepancy in the susceptibility category (60.4% highly susceptible by the E test versus 9.5% by the agar dilution assay). However, this observation is limited by the difficulty in assessing gentamicin susceptibility categories. For 77% of the 1996 *N. gonorrhoeae* isolates from Malawi, the MICs were within 1 dilution on both tests.

This low rate of agreement between the E test and agar dilution may be at least partially attributed to the performance of the E test in a field setting. Previous comparisons were performed in the same reference laboratory. In a field laboratory, the difficulties in achieving a consistent inoculum density for the E test may have contributed to the relatively low concordance between the two tests, since the 0.5 McFarland turbidity of the inoculum was determined by the naked eye against the ever-changing natural light from a window. In addition, the edges of the inhibition zone on each side of the strip were often not symmetrical, leaving the interpretation of the exact MIC to the laboratory technician. The use of a cotton-tipped swab rather than a wire loop to inoculate plates for the E test (and disc diffusion) in future trials may provide more even bacterial growth.

Despite all of the possible difficulties, the MICs for our *E. coli* control were consistently within the standard ranges. *E. coli* may not be the optimal control for tests using gonococcal suspensions, since the suspension characteristics of the organisms are different. However, the laboratory in Lilongwe, Malawi, was not equipped to maintain long-term cultures of an *N. gonorrhoeae* reference strain, and a control gentamicin susceptibility range has not been established for this organism. The *E. coli* culture was easily maintained in the field laboratory, and our achievement of consistent E-test and disc diffusion control MICs for this strain validates the drug delivery devices and agar plates used in this study.

Although disc diffusion is the most widely available and least expensive method studied, it was not helpful in determining the efficacy of gentamicin since there was very little difference in the range of disc diameters at different MICs determined by agar dilution. Gentamicin discs containing different drug concentrations may be more discriminatory between susceptible and resistant *N. gonorrhoeae* isolates. This possibility remains to be tested.

We found no single method of antimicrobial susceptibility testing that was sufficient when used alone for the surveillance of gonococcal susceptibility to gentamicin. The clinical efficacy data showed a trend toward increasing resistance to gentamicin, with cure rates of 95% in 1993 and 92% in 1996, but the difference was not statistically significant. As a method of monitoring susceptibility patterns, clinical efficacy testing is time-consuming and is biased by losses to follow-up. The high follow-up rate in this study was largely due to monetary stipends and adequate full-time staff hired specifically for the study, both of which would not be available for routine monitoring carried out by the government of Malawi. However, the bias due to loss to follow-up would be in the direction of an increased level of resistance since those with a poor response to treatment would be more likely to return to the clinic. The effect of bias due to reinfection between visits would also overestimate the degree of reinfection in clinical studies.

There was a general trend toward higher gentamicin MICs as determined by the agar dilution assay for 1996 *N. gonorrhoeae* isolates than for 1993 isolates. In addition, the percentage of isolates with high susceptibility (MIC, ≤ 4 μg/ml) decreased from 17.2 to 9.5% of the total isolates. This parallels the findings of the clinical study. Almost all of the treatment failures reported here were in the category of moderate susceptibility, but the two isolates in the low-susceptibility category responded to treatment with gentamicin. Since gentamicin is still effective for the treatment of gonorrhea in Malawi, we were not able to establish an MIC that clearly showed resistance.

As resistance to gentamicin increases, adoption of second-line antibiotics for the treatment of *N. gonorrhoeae* must be considered. There were no signs of resistance to the other antibiotics tested in this study. All isolates continued to be fully sensitive to ciprofloxacin, ofloxacin, and cefixime, largely due to their relative lack of availability in Malawi. However, the large price differential between gentamicin and the next line of antibiotics makes the decision to change treatment guidelines to a more effective drug a difficult one in the setting of an overall drug shortage.

There is a need for ongoing antibiotic susceptibility monitoring systems in resource-poor countries. While the agar dilution assay remains the method of choice for MIC determination in reference laboratories in developed countries, the E test could be operational within a developing country laboratory setting. However, the poor performance of the E test in this study suggests that its role is limited in the monitoring of antibiotic efficacy for gonorrhea under field conditions. It is possible that the E test would be a valuable tool for following trends in MICs, especially when used in combination with a clinical cure study. Further research under field conditions in developing countries using different antibiotics is required before the E test can be adopted as the sole tool for the monitoring of *N. gonorrhoeae* antibiotic susceptibility.

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