ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1987, p. 1744–1749
0066-4804/87/111744-06$02.00/0
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Vol. 31, No. 11

Effects of Different Media and Methods of Inoculum Preparation on Results of Antimicrobial Susceptibility Testing of *Neisseria gonorrhoeae* by Agar Dilution

JO-ANNE R. DILLON,[1]* WALTER TOSTOWARYK,[2] AND MARIELLE PAUZÉ[1]

Antimicrobials and Molecular Biology Division[1] and Bureau of Epidemiology,[2] Laboratory Centre for Disease Control, Ottawa, Ontario K1A OL2, Canada

Received 7 August 1987/Accepted 20 August 1987

The effects of two methods of inoculum preparation (the opacity standard method and a template method) and three different types of media on the penicillin, tetracycline, spectinomycin, and erythromycin MICs for 191 non-penicillinase-producing, 49 penicillinase-producing, and 5 tetracycline-resistant isolates of *Neisseria gonorrhoeae* were evaluated. Three World Health Organization reference strains (III, V, and VII) were similarly evaluated. Inoculum preparation method did not significantly alter the MIC (i.e., within a twofold dilution) of either susceptible or chromosomally resistant non-penicillinase-producing isolates; MICs achieved by the template method were slightly higher, but these differences were not significant. However, with penicillinase-producing and tetracycline-resistant isolates, the template method, which delivered 10^4 CFU, produced unequivocal MICs (denoting clinical resistance) which were significantly higher than MICs observed with the opacity standard method (inoculum, 10^3 CFU). With penicillin-, spectinomycin-, and erythromycin-containing medium, addition of hemoglobin to the medium produced lower, though not significantly different, MICs with all isolates as compared with MICs on medium without hemoglobin. Media supplemented with hemoglobin produced higher tetracycline MICs with all isolates, which were significantly different (greater than twofold) from MICs on the same hemoglobin-free media. Changes in auxotype did not alter overall observations concerning the effects of different media and inocula on MICs.

MIC testing methods recommended for most microorganisms are not generally applicable to *Neisseria gonorrhoeae* (7, 10). The international method recommended for gonococcal antimicrobial susceptibility testing by agar dilution differs in several respects from methods suggested by other groups, such as the National Committee for Clinical Laboratory Standards (7, 10). None of these methods have undergone rigorous methodological analysis. As a result of these conflicting and untested recommendations, considerable variation in the methods used for gonococcal susceptibility testing exists. For example, although supplementation of media with hemoglobin is recommended, in practice, several types of media, with and without hemoglobin, are commonly used (9, 10, 11, 13). The international recommended method of inoculum preparation for gonococcal susceptibility testing advocates that an inoculum of 5×10^3 CFU be delivered to the agar; by contrast, the National Committee for Clinical Laboratory Standards protocols recommend an inoculum of 10^4 CFU (7, 10). Since gonococci grow poorly in liquid culture, both methods advocate suspension of cells from solid medium in broth before adjustment of optical density. This procedure is inexact (gonococci tend to clump despite vigorous vortexing, and slower-growing strains or strains adhering to the agar surface are difficult to suspend), and the time involved in the preparation of large numbers of gonococcal strains may result in autolysis. In laboratories where MIC testing of *N. gonorrhoeae* is performed for the first time, inoculum preparation is the step most often improperly completed. Other variables which have not been standardized for gonococcal susceptibility testing by agar dilution include lot-to-lot variability of agar medium and standardization of commercial supplements added to media.

We examined two of the variables that affect gonococcal antimicrobial susceptibility tests: medium composition and inoculum preparation method. We compared both a clear medium (GC medium base supplemented with 1% defined supplement) and hemoglobin-supplemented media (GC medium supplemented with 1% defined supplement and 1% hemoglobin; Proteose no. 3 agar supplemented with 1% defined supplement and 1% hemoglobin) for ability to reproduce drug MICs for World Health Organization reference strains (III, V, and VII), susceptible and resistant non-penicillinase-producing isolates of *N. gonorrhoeae*, penicillinase-producing *N. gonorrhoeae* isolates, and isolates with plasmid-mediated resistance to tetracycline.

We also compared the effects of two methods of inoculum preparation on MIC. The opacity standard method, which is based on the adjustment of gonococcal suspensions to a barium sulfate opacity standard such that a final inoculum of 10^3 CFU is delivered, is the recommended international standard method (10). The template method, a method evaluated for the first time in this study, obviates many of the difficulties inherent with the opacity method, such as autolysis and clumping in solution, and difficulties in standardizing inoculum visually and delivers 10^4 CFU. With this method, strains were inoculated on agar over an area defined by a Steers replicator prong template. After incubation of the template overnight, the replicator prongs were pressed into

* Corresponding author.

the inoculum and samples were subsequently inoculated into the replicator wells.

MATERIALS AND METHODS

Bacterial isolates. The *N. gonorrhoeae* strains included in the study comprised 191 non-penicillinase-producing isolates, 49 penicillinase-producing isolates, 3 isolates with plasmid-mediated tetracycline resistance, and 2 with plasmid-mediated resistance to both tetracycline and penicillin. The isolates were originally submitted by provincial public health laboratories and made up part of the collection of clinical isolates in the Antimicrobials and Molecular Biology Division. They were confirmed as *N. gonorrhoeae* by using the criteria of colony morphology, Gram stain, oxidase reaction, β-lactamase production, and carbohydrate utilization (6). The nutritional requirements (auxotypes) of the non-penicillinase-producing isolates, determined by the method of Hendry and Stewart (3), were as follows: proline, citrulline, and uracil requiring (PCU^-; $n = 63$); nonrequiring (NR) or wild type ($n = 44$); ornithine requiring (O^-; $n = 7$); proline requiring (P^-; $n = 34$); ornithine, uracil, and hypoxanthine requiring (OUH^-; $n = 21$); citrulline, uracil, and hypoxanthine requiring (CUH^-; $n = 4$); $P^- O^-$ ($n = 2$); $POUH^-$ ($n = 5$); $O^- H^-$ ($n = 4$); $P^- H^-$ ($n = 2$); others ($n = 5$). The penicillinase-producing isolates included the following auxotypes: NR ($n = 25$); P^- ($n = 14$); $P^- O^-$, $P^- M^-$ (methionine), and $P^- L^-$ (leucine) ($n = 2$ each); O^- ($n = 3$); L^- ($n = 1$). Isolates with plasmid-mediated resistance to tetracycline were either NR (three, including two penicillinase-producing isolates) or P^- (two). The reference strains were World Health Organization strains III, V, and VII (8, 10). Before being tested, all isolates were stored at −70°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) plus 20% glycerol (1).

Media and antibiotics. Isolates were routinely subcultured on GC medium base (Difco) supplemented with 1% vol/vol of Kellogg defined supplement (1, 4, 7) and incubated at 35°C in a humid environment with 5% CO_2 for 18 to 24 h.

The following three media were compared for susceptibility testing: (i) GC medium base supplemented with 1% defined supplement (G medium); (ii) G medium plus 1% hemoglobin (GH medium); (iii) Proteose no. 3 agar (Difco) plus 1% defined supplement plus 1% hemoglobin (PH medium). Penicillinase-producing and non-penicillinase-producing isolates were tested on two different lots of media. Within each group of isolates, only a single lot of medium was used.

Antibiotic-containing media were prepared by adding appropriate volumes of antibiotic stock solution to the above media in doubling dilutions to produce the following range of concentrations: penicillin and ampicillin (Ayerst Laboratories, Montreal, Quebec, Canada), 0.008 to 512.0 mg/liter; spectinomycin (The Upjohn Co. of Canada, Don Mills, Ontario, Canada), 4.0 to 32.0 mg/liter; tetracycline hydrochloride (Bristol-Myers Pharmaceutical Group, Ottawa, Ontario, Canada), 0.063 to 64.0 mg/liter; erythromycin (Eli Lilly Canada Inc., Scarborough, Ontario, Canada), 0.032 to 4.0 mg/liter.

Preparation of inoculum and MIC testing. MIC tests comprised the agar dilution method (1, 7, 10), coupled with inoculation of antibiotic-containing medium with a Steers replicating device (14). Two methods of inoculum preparation were used. Method 1 was the template method. In a modification of a method used by Maier et al. (5), a single master plate (G medium) was inoculated with 32 different gonococcal isolates by spreading inoculum from 5 to 10 colonies collected on a sterile toothpick over an area defined by a template corresponding to the pattern of one of the Steers replicator prongs (14). The master plate was incubated for 18 h at 35°C in 5% CO_2. Subsequently, sterile Steers replicator prongs, held in place with the inoculating apparatus, were pressed onto the surface of the master plate and immersed immediately in the replicator block wells which contained 0.5 ml of 0.7% Casamino Acids (Difco). Three types of antibiotic-containing media, as described above, as well as antibiotic-free control media, were inoculated with the Steers replicating device.

Method 2 was the opacity standard method. With this internationally recommended method (10), which, up to 1987, was also used by the Centers for Disease Control, Atlanta, Ga. (Joan Knapp, personal communication), an overnight plate culture of *N. gonorrhoeae* is flooded with 3 ml of Trypticase soy broth. After gentle removal of cells from the agar surface with a sterile glass rod, pipetting of the suspension into a sterile test tube, and mixing with a Vortex mixer, the optical density of the suspension is visually adjusted to the opacity of a 0.5 McFarland barium sulfate standard. We modified this method by suspending a loopful of cells in 3.0 ml of 0.7% Casamino Acids, vortexing the cells, and then adjusting the optical density as described above. This suspension was further diluted (in 0.7% Casamino Acids) by 1:100 as recommended in the international methods. This process was repeated for each of the isolates, and Steers replicator wells were filled before inoculation of antibiotic-containing media and antibiotic-free control plates. Theoretically, an inoculum of approximately 1.2×10^3 cells was delivered if the Steers replicating device delivered 0.001 ml (7).

Test results were recorded after incubation for 18 h at 35°C in a humid environment supplemented with 5% CO_2. The MIC was considered to be that concentration of antibiotic that completely inhibited growth or allowed only one colony to grow. Tests with reference strains were repeated over time and served as an internal quality control for MIC testing.

Experimental design and analysis of antibiotic susceptibility tests. For the 191 non-penicillinase-producing isolates, a two-by-three factorial design was used for studying inoculum and medium effects on penicillin and tetracycline MICs with the two inoculum methods and the three media. A two-by-two factorial design was used with spectinomycin and erythromycin with the two inoculum methods and only two of the three media, namely, G and GH. Subsequently, the same experimental design was used in studying the effects of the two inoculum methods and the three media on the MICs of penicillin, spectinomycin, and tetracycline with the 49 penicillinase-producing isolates, as well as with the 5 tetracycline-resistant isolates.

Non-penicillinase-producing isolates were analyzed as a single group, as susceptible and resistant groups, and by auxotype. Because penicillinase-producing isolates were restricted to only a few auxotypes, analysis by auxotype was not undertaken with these strains.

Comparisons with different media and inoculum preparations were assessed on the basis of MIC ratios calculated for all isolates. For each antibiotic, the frequency distribution of the MIC ratios for each pairwise inoculum-medium combination was summarized, showing the ratio range and the percentage of ratios falling within 1 dilution step of unity. In addition, analyses of variance were conducted on the MIC data based on the $\log_2 + 10$ transformation (12). Because of the twofold dilutions used in the analytical work, the $\log_2$

transformation was used, and addition of the constant 10 was used to avoid negative values.

RESULTS

With the 191 non-penicillinase-producing isolates, the MIC (milligrams per liter) distributions for all inoculum-medium combinations (within a twofold dilution at either end of the spectrum) were as follows: penicillin, 0.008 to 8.0; tetracycline, 0.125 to 8.0; spectinomycin, 4.0 to 16.0; erythromycin, 0.032 to 8.0. Results of the pairwise comparisons between the effects of the different inoculum-medium combinations on the penicillin, tetracycline, erythromycin, and spectinomycin MICs for 191 non-penicillinase-producing isolates are summarized in Table 1. An MIC ratio of 1 indicated that the MICs tested under various inocula and media were identical. Results were also considered equivalent if the MIC ratio range extended from 2^{-1} to 2, i.e., within 1 dilution of unity. When the two inoculum methods were compared with different antibiotic-containing media (comparisons number 1 to 3 for penicillin and tetracycline and 1 and 2 for spectinomycin and erythromycin), over 96% of the MIC ratios for non-penicillinase-producing strains were in the 2^{-1}-to-2 ratio range. Thus, differences in methods of inoculum preparation did not produce significant changes in the MICs of any of the antibiotics tested. When inoculum comparisons were made with control strains (data not shown), 100% of the strains produced ratios within a twofold dilution difference for all of the antibiotic MICs.

For pairwise medium comparisons (numbers 4 to 9 for penicillin and tetracycline and 3 and 4 for spectinomycin and erythromycin), over 97% of the observations of the spectinomycin, erythromycin, and penicillin MIC ratios were within the 2^{-1}-to-2 ratio range (Table 1). Only 1% of the MIC ratios (14 of 1,146 observations) for the six pairwise medium comparisons (Table 1) fell outside this ratio range with penicillin, and none did so with spectinomycin and erythromycin. These results indicate that the effects of changes in medium type did not significantly affect the penicillin, spectinomycin, or erythromycin MICs. With tetracycline-supplemented media, however, a shift in the frequency distribution of the MIC ratios for the pairwise medium comparisons indicated that the composition of the medium affected the MICs. When GH medium was compared with G medium, only 73 to 75% of the strains fell within the 2^{-1}-to-2 ratio. In all, 11% of the MIC ratios (125 of 1,146 observations) for the six pairwise medium comparisons with tetracycline fell outside this ratio range. These changes were significant. The effects of different media on the MICs for non-penicillinase-producing isolates were reproduced with the control strains. When the MICs of penicillin, spectinomycin, and erythromycin were compared on different media (data not shown), 100% of the control strains had MIC ratios within the 2^{-1}-to-2 range. Once again, however, the G-GH comparisons gave MIC ratios outside of the 2^{-1}-to-2 range (7 of 144 observations) on tetracycline-supplemented media.

Plots of the geometric mean MIC of each antibiotic with each inoculum-medium combination for non-penicillinase-producing strains (Fig. 1) reflect the results in Table 1; that is, inoculum preparation method did not produce significant (i.e., a twofold difference) changes in the MIC of any antibiotic. However, the geometric mean MICs under all conditions were slightly higher when the template inoculum preparation method was used. In addition, differences in medium composition did not significantly change penicillin, spectinomycin, and erythromycin MICs. With tetracycline, however, in contrast to the other three antibiotics, MICs on GH medium were significantly higher than on G medium.

TABLE 1. Frequency distribution of MIC ratios of medium and inoculum comparisons of 191 non-penicillinase-producing *N. gonorrhoeae* isolates

Antimicrobial agent and comparison no.	Inoculum/medium comparisons for MIC ratio calculation[a]	Frequency distribution of MIC ratios						
		8^{-1}	4^{-1}	2^{-1}	1	2	4	8
Penicillin								
1	T-G/O-G			4	150	36	1	
2	T-GH/O-GH			2	137	50	2	
3	T-PH/O-PH			2	154	34	1	
4	T-G/T-GH			7	150	34		
5	O-G/O-GH			4	138	49		
6	T-G/T-PH			8	112	65	6	
7	O-G/O-PH			4	118	65	4	
8	T-GH/T-PH			11	129	49	2	
9	O-GH/O-PH			13	143	33	2	
Tetracycline								
1	T-G/O-G			2	127	60	2	
2	T-GH/O-GH			3	127	56	4	1
3	T-PH/O-PH			3	116	65	7	
4	T-G/T-GH		50	135	6			
5	O-G/O-GH	1	46	138	6			
6	T-G/T-PH		2	91	93	5		
7	O-G/O-PH			82	104	4	1	
8	T-GH/T-PH			2	56	120	12	1
9	O-GH/O-PH				47	132	11	1
Spectinomycin								
1	T-G/O-G			3	146	42		
2	T-GH/O-GH			4	134	50	3	
3	T-G/T-GH			2	127	62		
4	O-G/O-GH			1	116	74		
Erythromycin								
1	T-G/O-G			7	127	56	1	
2	T-GH/O-GH			7	136	45	1	2
3	T-G/T-GH			2	132	57		
4	O-G/O-GH			3	135	53		

[a] Inoculum abbreviations: T, template method; O, opacity standard method. Medium abbreviations: G, GC medium base–1% defined supplement; GH, G–1% hemoglobin; PH, Proteose no. 3 agar–1% defined supplement–1% hemoglobin.

The MIC ratio ranges were also examined for non-penicillinase-producing isolates which were classified as susceptible or resistant (MIC, ≧1.0 mg/liter) to penicillin, erythromycin, and tetracycline. The MIC ratios of isolates susceptible or resistant to penicillin and erythromycin were within the 2^{-1}-to-2 range, indicating that changes in medium or inoculum preparation method did not significantly alter the MICs within these groups. With over 90% of the isolates described as resistant, tetracycline MIC ratios were within the 2^{-1}-to-2 ratio range. However, with tetracycline-supplemented media, only 67 to 76% of susceptible (i.e., MICs of <1.0 mg/liter) isolates had drug MIC ratios within the 2^{-1}-to-2 range. That is, supplementation of medium with hemoglobin produced higher tetracycline MICs among susceptible isolates.

Identical analyses comparing the effects of inoculum preparation and medium on the MICs for the individual auxotypes of the 191 non-penicillinase-producing strains indicated that differences in auxotype did not alter previous observations.

The viable counts of non-penicillinase-producing isolates

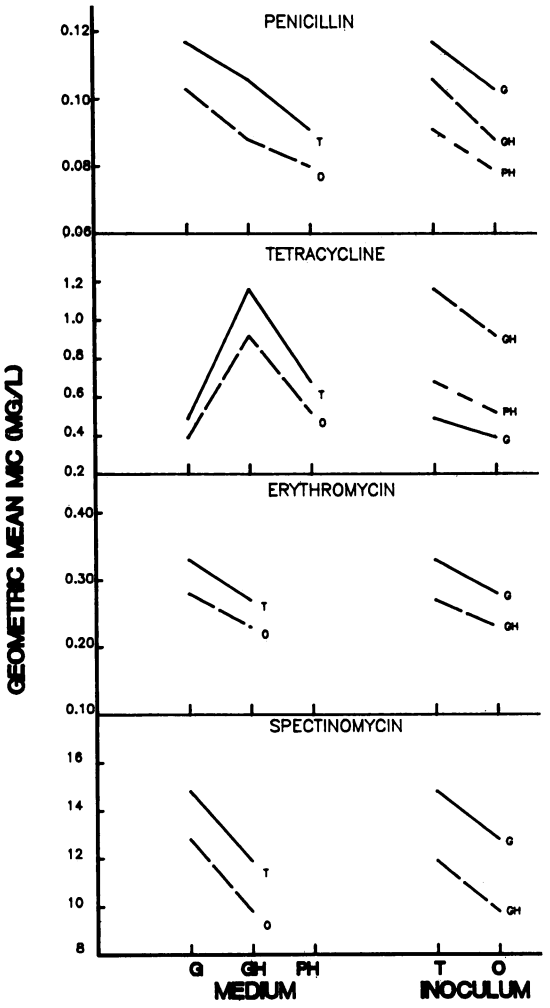

FIG. 1. Effect of various inoculum preparation methods and media on the geometric mean drug MICs for 191 non-penicillinase-producing isolates. See Table 1, footnote *a*, for inoculum and medium abbreviations.

prepared by both methods of inoculum preparation were determined. The viable counts of 23 strains standardized with the opacity standard method (calculated as an average of two samples) ranged from 1.2×10^9 to 1.0×10^6 CFU/ml, with an average of 1.5×10^8 CFU/ml. Thus, after dilution by 1:100 and since 0.001 ml (9) was delivered to an antibiotic-containing plate, the average final inoculum with the opacity standard method was 1.6×10^3 CFU/ml. The viable counts of 19 of the same strains by the template method of inoculum preparation (i.e., samples were taken from the replicator wells) ranged from 8.4×10^7 to 1.5×10^5 CFU/ml, with an average count of 2.0×10^7. The final inoculum delivered to the plates with a Steers replicator was, therefore, 2.0×10^4.

With the 49 penicillinase-producing isolates, the MIC (milligrams per milliliter) distributions for all inoculum-medium combinations (within a twofold dilution at either end of the spectrum) were as follows: tetracycline, 0.5 to 8.0; spectinomycin, 32.0. The penicillin MIC range with all media with the opacity standard inoculum preparation method was 2.0 to 32.0 mg/l, and with the template method the range was 8.0 to 256.0 mg/l. The geometric mean MICs of penicillin, tetracycline, and spectinomycin used to supplement media for penicillinase-producing strains, are given in Fig. 2. With spectinomycin, neither inoculum method nor medium composition affected the geometric mean MIC for penicillinase-producing isolates. For tetracycline MICs, no significant differences (i.e., twofold) were observed between inoculum preparation methods, although slightly higher MICs were produced by using the template method. As with non-penicillinase-producing isolates, tetracycline MICs for penicillinase-producing strains on media supplemented with hemoglobin were significantly higher than MICs on G medium; in addition, MICs on PH medium were higher than MICs on GH medium. With penicillin, different media did not alter the drug MICs for penicillinase-producing isolates; however, the template inoculum preparation method produced MICs which were significantly higher than those obtained with the opacity standard method.

Analysis of data for the reference strains indicated that differences between the geometric mean MICs from the two lots of G medium (non-penicillinase-producing strain study versus penicillinase-producing strain study) were within a twofold dilution for the three antimicrobial agents in which both lots were used. With the reference strains, the geometric mean MICs of the three antimicrobial agents for the non-penicillinase-producing strain study lot and the penicil-

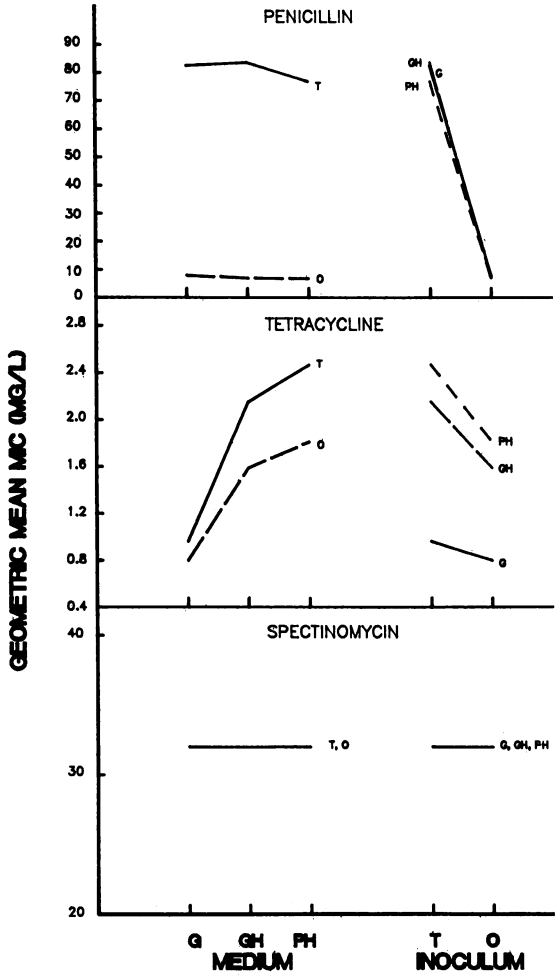

FIG. 2. Effect of various inoculum preparation methods and media on the geometric mean drug MICs for 49 penicillinase-producing isolates. See Table 1, footnote *a*, for inoculum and medium abbreviations.

linase-producing strain study lot, respectively, were as follows: penicillin, 0.13 versus 0.17 mg/ml; tetracycline, 0.42 versus 0.41 mg/ml; spectinomycin, 15.2 versus 29.3 mg/ml. Erythromycin was not examined in the penicillinase-producing strain study. Within each medium lot, there were no statistically significant differences ($P > 0.05$) among the repeats of the reference strains for each of the four antimicrobial agents used in the two studies.

Five isolates with plasmid-mediated tetracycline resistance were also analyzed on tetracycline- and spectinomycin-supplemented media. (Because three of these isolates also produced penicillinase, an analysis of inoculum-medium effects with penicillin is not described.) Inoculum-medium effects with tetracycline-resistant strains were similar to those found with penicillinase-producing strains (data not shown). That is, the spectinomycin MIC was unaltered by either inoculum preparation method or medium composition. Supplementation of tetracycline-containing medium with hemoglobin resulted in significantly higher tetracycline MICs than those observed with G medium. Furthermore, higher MICs were recorded with the template inoculum preparation method, particularly with hemoglobin-supplemented medium.

DISCUSSION

The present study shows that different inoculum preparation methods did not significantly (i.e., a twofold dilution difference) alter MICs for non-penicillinase-producing isolates. Hall and Opfer have also found that inoculum size had little influence on MICs for penicillinase-negative *N. gonorrhoeae* (2). The National Committee for Clinical Laboratory Standards (7) recommends that an inoculum of 10^4 be delivered to the antibiotic-containing plates in agar dilution tests. The template method, in fact, delivered such an inoculum, whereas the opacity standard method delivered approximately 90% fewer bacteria. These differences may explain why the template method produced slightly higher MICs than the opacity standard method. With penicillinase-producing isolates, a significant difference between inoculum preparation methods was observed for penicillin MICs. The lower inoculum delivered with the international recommended opacity standard method for testing penicillinase-producing strains can contribute to misleading conclusions regarding clinical resistance. With the template method, MICs for penicillinase-producing strains were significantly higher and unambiguous in terms of clinical resistance.

A variety of media have been used for MIC determinations with gonococcal isolates; we tested three of these media. Although no clinically significant differences in penicillin, erythromycin, or spectinomycin MICs were observed with different media, inclusion of hemoglobin in these media tended to lower the MICs of these antibiotics. The reverse was observed with tetracycline MICs; that is, MICs were significantly lower with hemoglobin-free medium. The reasons for these differences are not clear. Although divalent cations can inactivate tetracycline, thereby producing higher MICs, it is doubtful that this is the cause of the MIC changes in this study. Except for the addition of corn starch, the formula of GC medium base is similar to that of Proteose no. 3 agar; therefore, differences in MIC may be due to the binding or inactivation of tetracycline by the hemoglobin supplement. It is interesting to note that, among non-penicillinase-producing isolates, significant tetracycline MIC changes occurred with susceptible as opposed to resistant isolates. Clearly, further studies should be carried out to delineate these medium effects. The significant differences observed in tetracycline MICs with different media can give rise to clinical misinterpretations of the prevalence of resistant isolates. In addition, shifts in the prevalence of tetracycline-resistant isolates over time must be carefully analyzed, particularly if a new medium for MIC determination has been introduced.

In conclusion, we examined two variables which affect MICs for isolates of *N. gonorrhoeae*. It is clear that a more comprehensive examination of the factors which affect the MIC should be undertaken to recommend an effective, reproducible, internationally acceptable method. We found that the inoculum delivered by the present international recommended method was too low, thereby masking clinical resistance in penicillinase-producing isolates. We further conclude that the template method of inoculum preparation, an easier, less time-consuming method, delivers the appropriate inoculum and is an effective alternative. On the basis of results from the present study, we also propose that GC medium base replace the currently recommended hemoglobin-supplemented Proteose no. 3 agar for *N. gonorrhoeae* antimicrobial susceptibility testing.

ACKNOWLEDGMENT

We thank Surendranath Sanmugasunderam for the preparation of all graphic materials.

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