Tentative Criteria for Confirming the In Vitro Susceptibilities of Haemophilus influenzae and Neisseria gonorrhoeae to Two Fluoroquinolones (Sparfloxacin and Levofloxacin), Including Quality Control Parameters

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Sparfloxacin and levofloxacin were evaluated against 150 Haemophilus influenzae isolates and 149 Neisseria gonorrhoeae isolates in order to define susceptibility testing parameters. Sparfloxacin-susceptible H. influenzae strains were defined as those for which the MICs were \( \leq 0.25 \mu g/ml \) and the zones were \( \geq 30 \) mm, and N. gonorrhoeae susceptible strains were those for which the MICs were \( \leq 0.03 \mu g/ml \) and the zones were \( \geq 39 \) mm (5-\( \mu g \) disks). Levofloxacin-susceptible strains of H. influenzae included those for which the MICs were \( \leq 0.12 \mu g/ml \) and the zones were \( \geq 32 \) mm and N. gonorrhoeae susceptible strains were those for which the MICs were \( \leq 0.12 \mu g/ml \) and the zones were \( \geq 37 \) mm (5-\( \mu g \) disks). Criteria for a resistant category cannot yet be defined for either quinolone. In multilaboratory studies with different lots of Haemophilus Test Medium, replicate tests with the standard control strain of H. influenzae (ATCC 49247) were evaluated. For sparfloxacin disk tests, the proposed zone size limits were 33 to 42 mm and broth microdilution MIC limits were 0.004 to 0.016 \( \mu g/ml \), whereas for levofloxacin tests, zone size limits were 32 to 41 mm and broth microdilution MIC limits were 0.008 to 0.03 \( \mu g/ml \). Other multilaboratory studies evaluated tests with supplemented GC agar and N. gonorrhoeae ATCC 49226 for both drugs, zone size limits were 44 to 52 mm and agar dilution MIC limits were 0.004 to 0.016 \( \mu g/ml \).

Sparfloxacin and levofloxacin are fluoroquinolone compounds that are currently being evaluated in a number of clinical trials. Both drugs have broad spectrums of activity, including Haemophilus influenzae and Neisseria gonorrhoeae (2, 5–7). In the present report, we document the potencies of both drugs against H. influenzae and propose interpretive criteria for susceptibility tests performed with the Haemophilus Test Medium (HTM) described by Jorgensen et al. (11). In the same way, we evaluated tests of N. gonorrhoeae using supplemented GC agar (12, 13) in order to select interpretive criteria for agar dilution tests and for disk diffusion tests. In addition, we summarized the results of several multilaboratory studies that were designed to select quality control limits for dilution or disk diffusion tests with standard quality control strains.

MATERIALS AND METHODS

Antimicrobial-susceptibility tests were performed in accordance with the procedures outlined by the National Committee for Clinical Laboratory Standards (12, 13). For testing H. influenzae isolates, broth microdilution and disk diffusion tests were carried out with HTM broth and HTM agar which had been previously screened for proper performance with other antimicrobial agents (4, 12–14). For testing N. gonorrhoeae isolates, GC agar (Difco Laboratories, Detroit, Mich.) was used with an XV supplement devoid of cysteine (PML Microbiologicals, Tualatin, Oreg.). For agar diffusion susceptibility tests, 5-\( \mu g \) sparfloxacin disks and 5-\( \mu g \) levofloxacin disks were obtained from Becton Dickinson Microbiology Systems, Cockeysville, Md. (lot numbers 107530 and 202661, respectively). With either species, disk diffusion test plates were incubated at 35\(^\circ\)C in an atmosphere of 5 to 7% \( \text{CO}_2 \), as were agar dilution test mixtures with N. gonorrhoeae. Broth microdilution test mixtures with H. influenzae were incubated in ambient air.

MIC control limits were proposed as a result of four separate collaborative studies, each involving five independent laboratories. In two studies, replicate broth microdilution tests were performed with a standard control strain of H. influenzae (ATCC 49247). One study involved tests with sparfloxacin, and the other involved levofloxacin. In both cases, five laboratories each prepared microdilution trays with the study drug diluted in a different lot of HTM broth. A sixth lot of trays was shipped to all participants to provide a common lot control. Each of five laboratories performed 20 separate tests (different inocula) with the set of trays that they prepared and 5 separate tests with the common lot of trays with sparfloxacin or 10 tests with the common lot of trays with levofloxacin. That generated 125 sparfloxacin MICs and 150 levofloxacin MICs. The overall distribution of MICs generated by these exercises was evaluated, and control limits were selected to include MICs 1 doubling dilution interval on either side of the mode.

In the same way, replicate agar dilution tests were performed with N. gonorrhoeae ATCC 49226. Six separate lots...
generated 300 agar dilution MICs of each study drug. The overall distribution of the 300 MICs was then evaluated to select MIC quality control limits.

Additional collaborative studies were performed to establish zone size control limits for disk diffusion tests. Replicate disk tests were performed with *N. gonorrhoeae* ATCC 49226 on six different lots of GC agar from one of three manufacturers and with *H. influenzae* ATCC 49247 on six different lots of HTM agar. In both cases, one of the agar lots was common to all five investigators. Two lots of 5-µg sparflloxacin disks and two lots of 5-µg levofloxacin disks were tested each time. Each laboratory tested 30 separate inoculum preparations (20 on the unique lot of agar and 10 on the common lot of agar). This generated 60 zone size determinations by each participant, or a total of 300 zone diameters. The statistic described by Gavan et al. (10) was applied to calculate tentative control limits. That statistic assumes that the median zone for all 300 determinations is the idealized target value and that the extent of acceptable variation on either side of that target value is taken as the median of the five individual laboratory ranges rounded up to the next even value. We also applied an alternative statistic which has been used at the Clinical Microbiology Institute for several years. This uses the all-laboratory mean as the target value, and the range is based on the average of five individual laboratory standard deviations. In both cases, acceptable variability is based on intralaboratory ranges or standard deviations and does not involve interlaboratory variability. Interlaboratory differences influence only the idealized target value; a few aberrant test results in such dataset influence the means more than the medians.

Correlations between zone diameters and MICs were studied at the Clinical Microbiology Institute. Results from

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**FIG. 1.** Broth microdilution MICs for *H. influenzae* compared with zones of inhibition around 5-µg disks, all tested with HTM broth or agar. Vertical and horizontal lines represent proposed interpretive criteria for confirming susceptibility of *H. influenzae* isolates to sparflloxacin or levofloxacin.

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**FIG. 2.** Agar dilution MICs for *N. gonorrhoeae* compared with zones of inhibition around 5-µg levofloxacin or 5-µg sparflloxacin disks. Vertical and horizontal lines represent proposed interpretive breakpoints for identifying susceptible categories. The 23 strains for which the MICs were elevated and for which the zone diameters were smaller were laboratory-selected variants with relative resistance to both fluoroquinolones.
broth microdilution and disk diffusion tests were compared for 150 *H. influenzae* isolates, including 89 ampicillin-susceptible, 41 β-lactamase-producing ampicillin-resistant, and 20 β-lactamase-nonproducing ampicillin-resistant isolates. Ampicillin resistance was unrelated to fluoroquinolone susceptibility. All study strains were originally clinical isolates gathered from different U.S. medical centers.

Zone diameters and agar dilution MICs were compared for 149 isolates of *N. gonorrhoeae*, including 23 laboratory-trained strains that had been serially exposed to subinhibitory concentrations of different fluoroquinolones (1, 8, 9). The latter strains have shown a decreased susceptibility to all fluoroquinolones that have been tested to date, and that relative resistance has been found to be stable for at least 2 years. The remaining 126 gonococci were originally clinical isolates that included 22 β-lactamase-producing strains, 28 penicillin-resistant β-lactamase-nonproducing strains, and 76 penicillin-susceptible strains. Penicillin resistance was unrelated to fluoroquinolone susceptibility.

**RESULTS**

Scattergrams that compare broth microdilution MICs and disk diffusion tests for 150 *H. influenzae* isolates are presented in Fig. 1. Clinical isolates or laboratory-trained strains of *H. influenzae* with decreased susceptibilities to fluoroquinolone compounds were not yet available for testing; all 150 isolates were uniformly susceptible to both study drugs. The MICs of sparfloxacin were ≤0.125 μg/ml, and the MICs of levofloxacin were ≤0.06 μg/ml. For reasons that were outlined elsewhere (3), interpretive criteria were selected to describe the susceptible populations. In the absence of resistant or relatively resistant clinical isolates, a resistance category could not be defined. Following the guidelines that we previously used for selecting interpretive breakpoints for other fluoroquinolones (3), proposed sparfloxacin breakpoints are MICs of ≤0.25 μg/ml or zones of ≥30 mm (5-μg disks) for the susceptible category. For 5-μg levofloxacin disks, susceptible strains were those for which the zones were ≥32 mm in diameter or the MICs were ≤0.12 μg/ml. In the future, some strains may fail to meet these criteria for susceptibility. Such strains are not necessarily resistant; however, if they do occur, their decreased susceptibility needs to be confirmed by additional in vitro tests, and the patient’s response to chemotherapy needs to be documented.

Similar studies were performed with 149 *N. gonorrhoeae* isolates (Fig. 2). For the 23 laboratory-selected variants, the MICs of sparfloxacin were ≥0.06 μg/ml and those of levofloxacin were ≥0.25 μg/ml. For the 126 clinical isolates of gonococci, the zones were larger and the MICs were lower than those for the laboratory-selected variants. It seems reasonable to assume that all fresh clinical isolates of *N.
gonorrhoeae should be inhibited by ≤0.03 μg of sparfloxacin per ml and that zones around 5-μg sparfloxacin disks should be ≥39 mm in diameter. Furthermore, the MICs of levofloxacin should be ≤0.12 μg/ml, and zones around 5-μg levofloxacin disks should be ≥37 mm in diameter. In the future, clinical isolates that perform like our laboratory-selected variants might appear endemically. If they do occur, their responsiveness or lack of responsiveness must be documented before an appropriate interpretive category can be assigned. The interpretive criteria defined above identify only the normal population of gonococci that we now know, and we are assuming that they will be susceptible clinically.

To help standardize methodologic details, quality control parameters must be defined. The reproducibility of broth microdilution MICs with H. influenzae ATCC 49247 is described in Fig. 3. The all-laboratory modal MICs of sparfloxacin and levofloxacin were 0.008 and 0.016 μg/ml, respectively. Vertical dotted lines in Fig. 3 describe proposed control limits which include the mode ± 1 doubling dilution interval. All levofloxacin MICs and 91% of the sparfloxacin MICs fell within the proposed control limits. All of the 150 sparfloxacin MICs fell into a 4-dilution range (0.002 to 0.016 μg/ml), but the proposed 3-dilution range should reflect more realistic limits for quality control purposes. The results of replicate agar dilution tests with N. gonorrhoeae ATCC 49226 are displayed in Fig. 4. The all-laboratory mode for both drugs was 0.008 μg/ml, and the proposed control limits (0.004 to 0.016 μg/ml) included all of the 300 levofloxacin MICs and 297 of the 300 sparfloxacin MICs.

Replicate disk diffusion tests with H. influenzae ATCC 49247 were performed in five laboratories. Table 1 describes the results of this exercise. The overall arithmetic mean zone diameter was accepted as the theoretical target value. The average of five individual laboratory standard deviations was accepted as a reasonable extent of intralaboratory variation. The mean ± 2× the average standard deviation described the calculated range. The statistic described by Gavan et al. (10) was also applied; the ranges were 1 mm narrower because of the rounding-off process. To avoid excessively stringent control limits for tests on HTM agar, it has been common practice to arbitrarily subtract or add 2 mm at the calculated upper or lower limit, respectively. When that adjustment was arbitrarily applied, the zone size limits for 5-μg sparfloxacin disks were 33 to 42 mm in diameter, and that included 86% of all of our data (99% were within a range of 31 to 43 mm). For 5-μg levofloxacin disks, the adjusted limits were 32 to 41 mm, and 94% of our determinations fell within those limits (99% were within a 31- to 42-mm range). For other fluoroquinolone compounds which utilize a 5-μg disk, approved control limits span an 8- or 9-mm range (14). The 9-mm ranges proposed for the two study drugs seem to be realistic compromises, even though more stringent limits could be justified statistically and even broader ranges would be needed to include >95% of our collaborative data.

Table 2 describes the disk test data generated with N. gonorrhoeae ATCC 49226. One participant (Laboratory D) reported extremely variable and unusually large zones of inhibition. The same institution participated in exercises with the H. influenzae control strain (Laboratory E in Table 1) and tended to record zones slightly larger than those reported by others. Those participants were told of the problem with their tests of N. gonorrhoeae ATCC 49226, and additional disk tests were then performed with their test
TABLE 2. Results of a five-laboratory collaborative study of levofloxacin and sparfl oxacin disk susceptibility tests of N. gonorrhoeae ATCC 49226 tested on supplemented GC agar (six lots)

<table>
<thead>
<tr>
<th>Zone diam (mm)</th>
<th>Levofoxacin (5-μg disks)</th>
<th>Sparfloxacin (5-μg disks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A B C D E All</td>
<td>A B C D E All</td>
</tr>
<tr>
<td>43</td>
<td>3 2 5 3 12</td>
<td>7 2 12 7 11</td>
</tr>
<tr>
<td>44</td>
<td>12 10 24</td>
<td>10 20</td>
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<tr>
<td>45</td>
<td>17 10 29</td>
<td>20 40</td>
</tr>
<tr>
<td>46</td>
<td>13 30 48</td>
<td>5 4 20 29</td>
</tr>
<tr>
<td>47</td>
<td>8 11 6 25</td>
<td>10 3 0 16</td>
</tr>
<tr>
<td>48</td>
<td>19 3 6 24</td>
<td>15 7 0 22</td>
</tr>
<tr>
<td>49</td>
<td>10 9 1 24</td>
<td>15 7 0 22</td>
</tr>
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<td>50</td>
<td>9 1 20 9 10</td>
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<tr>
<td>51</td>
<td>7 3 0 10 5 5</td>
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</tr>
<tr>
<td>≥60</td>
<td>7 7 7</td>
<td>7 7</td>
</tr>
</tbody>
</table>

Median 51 48 45 56 46 48b 50 48 45 55 45 48b

Range 7 7b 6 14 6 7b 8 5 16 5

* Laboratories A to E each recorded 80 zone diameters, 40 on the assigned lot of GC agar and 20 on a control lot common to all five participants. The results obtained with both lots of agar are combined in this table because they did not differ greatly.

For both drugs, the overall median zone diameter was 48 mm, and that was assumed to be the theoretical target value. The range of acceptable variation in zone sizes was based on the median of the five individual laboratory ranges (7 mm for both drugs). By rounding up to the next even value, an 8-mm range (44 to 52 mm) was calculated for both disks.

lot of GC agar. The same aberrant results were recorded, and no obvious explanation could be found for the tendency to read somewhat larger zone diameters. Data from all five laboratories were analyzed, and that analysis was repeated after exclusion of all data reported by Laboratory D. Both analyses led to identical conclusions. The statistic described by Gavan et al. (10) was utilized because median values were not markedly affected by aberrant results such as those reported by Laboratory D. Nearly identical (±1-mm) control limits were calculated as the all-laboratory mean zone diameter ± 2× the average of 5 or 4 individual laboratory standard deviations. We tentatively propose limits of 44 to 52 mm for both drugs. For the two study drugs, Laboratory D reported that 100 of 120 zones exceeded the 52-mm upper limit, and that should have warned the workers at that laboratory that there was a problem that needed to be resolved. Some investigators now feel that the zone size limits should include at least 95% of all zones recorded by the five participants. To conform to such a rule of thumb, sparfloxacin control limits should be 43 to 56 mm (13-mm range) and levofloxacin limits should be 44 to 58 mm (14-mm range). The proposed 8-mm ranges (44 to 52 mm) are consistent with the control limits that are currently being used for other fluoroquinolones with 5-μg disks (14).

DISCUSSION

Clinical isolates of H. influenzae and N. gonorrhoeae were uniformly susceptible to sparfloxacin and to levofloxacin. Consequently, we could describe only breakpoints that would identify the normal population that is currently being observed. That population may change with continued use of the fluoroquinolones, and interpretive criteria for in vitro tests will have to be reevaluated as the changes occur. We cannot determine whether clinically resistant strains will be detected by disk diffusion or antibiotic dilution tests, but we are reassured to learn that laboratory-selected gonococci were not susceptible by either test procedure. The normal populations of H. influenzae and of N. gonorrhoeae isolates are both categorized as being susceptible to the two study drugs on the assumption that ongoing clinical trials will document the effectiveness of both drugs in treating gonorrhea and infection due to H. influenzae. Strains that fail to respond clinically should be studied carefully to determine whether they display any unique in vitro characteristics that distinguish them from the normal susceptible strains. Also, clinical isolates which are not susceptible by the proposed breakpoints should be evaluated carefully; they are not necessarily resistant clinically, but their clinical responsiveness or lack of responsiveness needs to be documented before they can be properly categorized.

The interpretive criteria and quality control limits presented in this report should be useful to those who are interested in performing in vitro tests to support clinical trials with either one of the fluoroquinolone compounds that we evaluated. The proposed interpretive criteria might need to be modified as clinical experiences are gathered. For that reason, our recommendations should be considered tentative at this point.

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