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Interpretive Criteria and Quality Control Parameters for Testing Susceptibility of *Haemophilus influenzae* to Enoxacin, Ofloxacin, and Temafloxacin

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Haemophilus influenzae **isolates were uniformly susceptible to enoxacin, ofloxacin, and temafloxacin. Zone diameter and MIC interpretive criteria were proposed to define susceptible populations so that mutants with diminished susceptibility might be detected when and if they appear in clinical specimens. Additional collaborative quality control studies defined MIC and zone size limits for tests with *H. influenzae* ATCC 49247.**

The fluorinated quinolone compounds represent a large number of broad-spectrum antimicrobial agents with potent antibacterial activity against a variety of species, including *Haemophilus influenzae*. Procedures for testing the susceptibility of *H. influenzae* to various antimicrobial agents have been standardized. The Haemophilus Test Medium (HTM) of Jorgensen et al. (7) has been adopted (8, 9) with incubation in 5 to 7% CO_2 (agar media) or in ambient air (broth media). Because procedures used to test *H. influenzae* differ from those used for other microorganisms, interpretive criteria and quality control parameters specific for tests with *H. influenzae* are needed. In this report, we describe efforts to develop such criteria for testing of three fluoroquinolones (enoxacin, ofloxacin, and temafloxacin) against *H. influenzae*. Multilaboratory studies were also carried out to define quality control parameters for testing of *H. influenzae* ATCC 49247 by disk diffusion and by broth microdilution methods.

Susceptibility tests with 10-μg enoxacin disks were evaluated first by testing 100 isolates of *H. influenzae* (60 ampicillin-susceptible and 40 ampicillin-resistant strains, including 20 β-lactamase-negative strains). Subsequent evaluation of tests with 5-μg ofloxacin disks and 5-μg temafloxacin disks involved 150 strains (89 ampicillin-susceptible and 61 ampicillin-resistant strains, including 20 β-lactamase-negative strains). Throughout these studies, the testing procedures used were those described by the National Committee for Clinical Laboratory Standards (8, 9). Disk diffusion and broth microdilution tests were performed with different lots of HTM that have been found to be satisfactory when standard control strains were tested against a variety of other antimicrobial agents with established performance criteria (1, 6, 8, 9).

For quality control studies, replicate broth microdilution susceptibility tests with *H. influenzae* ATCC 49247 were performed by five different collaborating facilities. The participants each prepared a different lot of microdilution trays, and a sixth control lot was distributed to all laboratories for additional testing. All participants generated 20 MICs each with separate inoculum preparations by using their own microdilution trays, and another set of 5 MICs was generated with the control lot common to all investigators. The inoculum density was confirmed by performing viable cell counts with samples removed from freshly inoculated growth control wells in randomly selected trays. Each of five participants performed five separate colony counts, and the 25 inocula averaged 7×10^5 (range, 1×10^5 to 3×10^6) CFU/ml. Despite the apparent wide range of inocula, MICs were quite reproducible (Table 1). All MICs of enoxacin and ofloxacin and 97% of the temafloxacin MICs were no more than 1 doubling dilution on either side of the mode (Table 1). The MIC control limits were calculated from these data (2). These limits are for broth microdilution tests only; they may or may not be useful for monitoring of agar dilution tests. An alternative control strain of *H. influenzae* (ATCC 49766) was not included, since it was not needed for monitoring of these drugs (3, 6).

Three different lots of disks were also evaluated by five collaborating laboratories, each using a different lot of HTM agar plus a control lot common to all participants. All six lots of HTM agar supported growth of *H. influenzae* ATCC 10211 as well as ATCC 49247. Zone size limits were defined by the statistic of Gavan et al. (5). When enoxacin and temafloxacin disks were tested on HTM agar incubated in 5 to 7% CO_2, there were no significant differences between laboratories or between HTM agar lots (Table 2). With ofloxacin disks, one laboratory reported unusually large zones of inhibition on the control lot of agar as well as on the unique lot assigned to that laboratory. The aberrant ofloxacin disk test results reported by that one laboratory remain unexplained and were excluded from Table 2. The calculated control limits for ofloxacin disk tests excluded 58% of the determinations recorded by that laboratory. The other laboratories reported zones that were within the recommended zone size limits or no more than 1 mm outside of the limits (one zone was 2 mm beyond the upper limit).

All of the *H. influenzae* isolates that were tested in these studies were exquisitely susceptible to the three fluoroqui-

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TABLE 1. Distribution of broth microdilution MICs reported by five independent laboratories in a coordinated study involving replicate tests with *H. influenzae* ATCC 49247

MIC (μg/ml)	Frequency with which each MIC was reported[a]		
	Enoxacin	Ofloxacin	Temafloxacin
0.004			2
0.008			7
0.016		1	76
0.03		99	38
0.06	7	25	2
0.12	81		
0.25	35		
0.5			

[a] Horizontal lines designate upper and lower limits of acceptable performance.

nolones, i.e., all were inhibited by concentrations well below the ≤2.0-μg/ml breakpoint concentration that has been applied to tests with other species (9, 10). Also, zones of inhibition were much larger than those currently used to define the susceptibility of other species (8, 10). Interpretive breakpoints that have been defined on the basis of expected concentrations in blood and tissue could be applied to tests with *H. influenzae*. In that case, we would only conclude that disk tests with all three agents give no discrepant results.

MICs for the bacterial populations that were studied were very uniform in distribution, and there was no evidence of relative or absolute resistance. There is no reason to believe that any one strain in these unimodal populations differs significantly from the others. Figure 1 displays the spread of MICs and zone diameters for each of the three fluoroquinolones. After extended use of these agents in clinics, one might expect to see strains with gradually increasing MICs and decreasing zones of inhibition. This anticipated decreased in vitro susceptibility may or may not represent

TABLE 2. Distribution of zone diameters reported by five independent laboratories, all of which tested *H. influenzae* ATCC 49247 on different lots of HTM

Zone diam (mm)	Frequency with which each zone was reported[a]		
	Enoxacin (10-μg disk)	Ofloxacin[b] (5-μg disk)	Temafloxacin (5-μg disk)
27			
28	2		
29	9		
30	88	7	
31	123	48	
32	160	110	
33	116	120	15
34	107	130	111
35	146	77	145
36	42	66	92
37	26	56	65
38	5	45	18
39	1	1	5
40			

[a] Horizontal lines designate upper and lower limits for acceptable performance.

[b] Ofloxacin data from one of the five investigators are not shown (58% of those excluded zones were outside of the control limits, i.e., ≥38 mm).

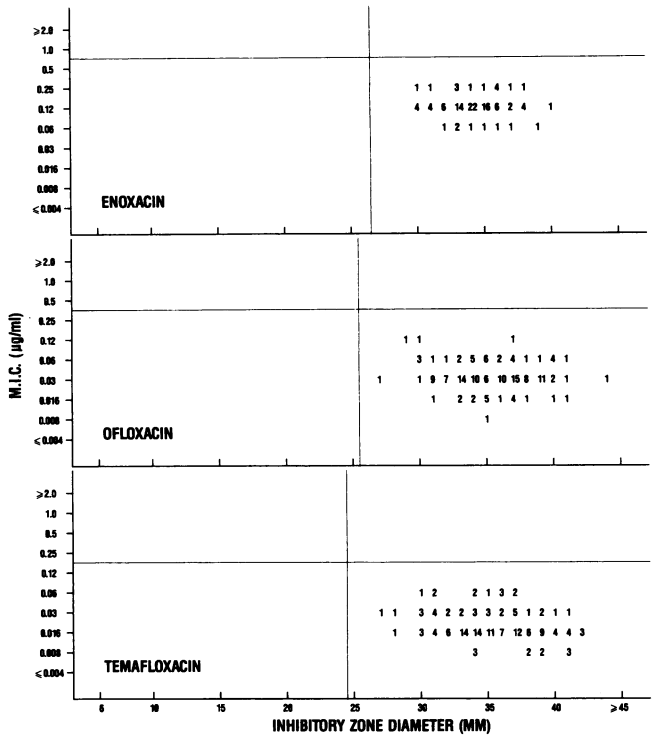

FIG. 1. Scattergrams displaying the spread of susceptibility test results obtained with *H. influenzae* isolates tested against enoxacin, ofloxacin, and temafloxacin. Horizontal and vertical lines represent proposed breakpoints for defining the susceptibility category for each fluoroquinolone: a resistance category cannot be defined.

development of strains that might be associated with diminished clinical cure rates. If the interpretive criteria that were developed for other microorganisms were also applied to tests with *H. influenzae*, mutants with slightly decreased susceptibility would probably be categorized as susceptible. Instead, we prefer to define an interpretive criterion that would identify those mutants that differ from the normal susceptible population. Identical circumstances were encountered when testing *Neisseria gonorrhoeae* against fluoroquinolone compounds, and similar criteria were applied (1, 4).

In the absence of a resistant population that can be included in our in vitro studies, we can define only a susceptible interpretive category for *H. influenzae*. Strains that fall outside of that population are not necessarily resistant clinically, but it seems prudent that they be designated for further evaluation when and if they appear. We arbitrarily selected an interpretive criterion that was based on the MIC for 99% of the strains tested or on the smallest zone produced by 99% of the susceptible strains. The susceptible population was then defined to include strains with MICs no more than 1 doubling dilution greater than the MIC for 99% of the strains or zones of inhibition no more than 3 mm smaller than the zone size for 99% of the strains. For all three drugs, those limits include 100% of the *H. influenzae* strains tested, not just 99% of the strains. With that system, susceptible strains with a four- to eightfold increase in MICs should be designated as no longer belonging to the susceptible population that we now know, but they would probably be susceptible by the criteria applied to other species. Whether the disk diffusion or broth microdi-

TABLE 3. Summary of recommended standards for testing of susceptibility of *H. influenzae* isolates to three fluoroquinolones

Drug (disk content [μg])	Zone diam (mm), MIC (μg/ml)	
	Interpretive criteria for susceptibility category	Quality control limits for *H. influenzae* ATCC 49247
Enoxacin (10)	≥27, ≤0.5	30–36, 0.06–0.25
Ofloxacin (5)	≥26, ≤0.25	31–37, 0.016–0.06
Temafloxacin (10)	≥25, ≤0.12	33–38, 0.008–0.03

lution tests will be capable of detecting such mutants remains to be seen. Furthermore, how those strains respond to chemotherapy must be determined before the proposed interpretive criteria can be assessed. By using the conservative criteria that we propose for testing of fluoroquinolones against *H. influenzae* isolates, the disk test can only confirm susceptibility: moderately susceptible or resistant categories have not been defined.

In summary, interpretive criteria and quality control limits that were proposed as a result of these studies are outlined in Table 3. After completion of this report, we learned that temafloxacin has been withdrawn from the market. The principles that were used to analyze these data are appropriate for the testing of most other fluoroquinolone compounds.

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