

Variation in Erythromycin and Clindamycin Susceptibilities of *Streptococcus pneumoniae* by Four Test Methods

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Susceptibilities of 124 strains of *Streptococcus pneumoniae* to erythromycin and clindamycin were determined by the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method, with incubation for 20 to 24 h in ambient air and with modifications of this method by incubation for up to 48 h in air and CO₂. Strains were also tested by agar dilution, E-test, and disk diffusion; good correlation was obtained with these methods, with clear separation into bimodal populations of susceptible and resistant strains. The broth microdilution method, however, using incubation in air for 24 h (NCCLS method), misclassified 4 of 92 erythromycin-resistant strains (1 as susceptible and 3 as intermediate) and 25 of 58 clindamycin-resistant strains (all as susceptible). With the exception of one strain with clindamycin, susceptible and resistant strains were correctly classified by the microdilution method with incubation in CO₂ for 24 h or in ambient air for 48 h. Disk diffusion, agar dilution, and E-test methods with incubation in 5% CO₂ are therefore reliable methods for susceptibility testing of pneumococci against these agents. However, the NCCLS microdilution method, which specifies incubation for 20 to 24 h in ambient air, produced significant very major errors (43%) with clindamycin. Modification of the microdilution method by incubation in 5% CO₂ or by extension of incubation time in ambient air to 48 h corrected these errors. Disk diffusion, however, was shown to be a simple, convenient, and reliable method for susceptibility testing of pneumococci to erythromycin and clindamycin and is suggested as the method of choice for these agents.

Streptococcus pneumoniae is the most common causative organism of community-acquired pneumonia, bronchitis, otitis media, and sinusitis. Pathogens in these diseases are rarely identified due to lack of adequate clinical specimens, and antimicrobial therapy is usually chosen empirically. Erythromycin and other macrolides have been considered as belonging to one of the antibiotic groups of choice for treating many of these community-acquired infections because they are active against pneumococci and other pathogens commonly associated with these diseases (16). Macrolides are often the agents of choice for patients allergic to penicillin. However, erythromycin-resistant strains of *S. pneumoniae* have been isolated with increased frequency in several countries in Europe, as well as in Japan, Korea, Hong Kong, South Africa, and more recently also in the United States (1, 2, 4, 5, 8-12), and the choice of agents for empiric use needs to be reevaluated.

Erythromycin is a macrolide antibiotic which inhibits protein synthesis by binding to the 50S ribosomal subunit (19). Cross-resistance among macrolides, lincosamides, and streptogramin type B agents usually occurs in gram-positive organisms (11). Therefore, erythromycin-resistant strains of *S. pneumoniae* have usually been considered resistant to all other macrolides and lincosamides, including clindamycin, clarithromycin, and azithromycin (1, 7, 9). However, more recent reports have documented erythromycin-resistant *S. pneumoniae* strains in the United States that are susceptible to clindamycin (3, 15), and the same phenomenon has been reported for *S. pyogenes* in Finland (17).

While testing erythromycin and clindamycin against pneu-

mococci by broth microdilution using ambient air incubation for 20 to 24 h as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for susceptibility testing of pneumococci (13), we observed many isolates that showed trailing endpoints after incubation, particularly with clindamycin, although this was also noted with some isolates with erythromycin. These observations prompted a study to compare methods for susceptibility testing of macrolides and lincosamides against pneumococci.

Four methods for susceptibility testing of *S. pneumoniae* with erythromycin and clindamycin were evaluated: broth microdilution MIC, agar dilution MIC, E-test MIC, and disk diffusion testing. Test variables used were incubation in ambient air compared to incubation in 5% CO₂ and extension of incubation time from 24 to 48 h.

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MATERIALS AND METHODS

Strains. One hundred and twenty-four selected strains of *S. pneumoniae* were chosen from our pneumococcal collection to represent as many countries, resistance patterns (other than resistance to macrolides), and serotypes as possible, with an emphasis on including as many erythromycin-resistant strains as possible. The final selection included 92 erythromycin-resistant strains with 32 erythromycin-susceptible strains chosen as controls. Strains were all clinical isolates recovered from various sources representing invasive and noninvasive strains, from the United States, Europe, Africa, Japan, and Korea.

Susceptibility testing. Antimicrobial agents. The following agents were used: penicillin G (Squibb Institute for Medical Research, Princeton, N.J.), erythromycin (Abbott Laboratories, North Chicago, Ill.), and clindamycin (Upjohn Co., Kalamazoo, Mich.).

Quality control organisms. *S. pneumoniae* ATCC 49619 was tested by all methods on each day of testing. Additional control strains included *Staphylococcus aureus* ATCC 29213 for broth microdilution and agar dilution testing and *S. aureus* ATCC 25923 for disk diffusion testing.

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Broth microdilution MICs. Microdilution trays were prepared in house using cation-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) with 5% lysed horse blood (Cleveland Scientific, Bath, Ohio). Erythromycin and clindamycin were tested at doubling dilutions from 0.03 to 32 $\mu\text{g/ml}$. Inocula were prepared by suspending growth obtained from sheep blood agar plates that had been incubated for 20 to 24 h in 5% CO_2 . Colonies were suspended in Mueller-Hinton broth to obtain a suspension equivalent to a 0.5 McFarland turbidity standard. The suspension was further diluted within 15 min to provide a final inoculum density of 5×10^5 CFU/ml. Trays were inoculated in duplicate, with one being incubated aerobically as recommended by the NCCLS (13) and the other being incubated in 5% CO_2 . MICs were read at 24 and 48 h. Trays were not stacked when incubated in the 5% CO_2 atmosphere to allow adequate exposure of the tray to the atmospheric conditions. Inoculum size was checked by performing viable counts of inocula on random strains throughout the study for all test methods.

Agar dilution MICs. Agar dilution plates were made by adding antibiotic solutions to molten Mueller-Hinton agar (Difco) supplemented with 5% sheep blood. Penicillin was tested at doubling dilutions from 0.008 to 32 $\mu\text{g/ml}$. Erythromycin and clindamycin were tested at doubling dilutions from 0.03 to 64 $\mu\text{g/ml}$. A multipoint replicator (Craft Machine, Chester, Pa.) was used to inoculate duplicate sets of agar plates with 1 μl of a suitably diluted organism suspension, prepared from suspensions equivalent to a 0.5 McFarland standard, to produce inocula of 10^4 CFU/spot. Plates were incubated aerobically and in 5% CO_2 , and MICs were read at 24 and 48 h.

E-test MICs. Erythromycin and clindamycin E-test strips (AB Biodisk, Piscataway, N.Y.), calibrated to read MICs from 0.016 to 256 $\mu\text{g/ml}$, were tested on 150-mm-diameter plates containing Mueller-Hinton agar supplemented with 5% sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Inocula were prepared as described above for the agar dilution. Plates were incubated in 5% CO_2 , and MICs were read at 24 and 48 h.

Disk diffusion. Disk diffusion tests were performed using 150-mm-diameter plates containing Mueller-Hinton agar supplemented with 5% sheep blood agar and inocula were prepared as for the E-tests above according to NCCLS methods (14). Antibiotic disks (Becton Dickinson) tested were erythromycin (15- μg disks), clindamycin (2- μg disks), clarithromycin (15- μg disks), and azithromycin (15- μg disks). Plates were incubated in 5% CO_2 , and zones of inhibition were read at 24 and 48 h.

Interpretative criteria for susceptibility testing. The MIC breakpoints published by NCCLS in M100-S6 of December 1995 (13) were used and were as follows: penicillin, ≤ 0.06 $\mu\text{g/ml}$, susceptible, 0.1 to 1 $\mu\text{g/ml}$, intermediate, and ≥ 2 $\mu\text{g/ml}$, resistant; erythromycin and clindamycin, ≤ 0.25 $\mu\text{g/ml}$, susceptible, 0.5 $\mu\text{g/ml}$, intermediate, and ≥ 1 $\mu\text{g/ml}$, resistant. The criteria used for interpretation of disk diffusion zones were also from NCCLS M100-S6 as follows: susceptible, ≥ 21 mm, and resistant, ≤ 15 mm for erythromycin and clarithromycin; susceptible, ≥ 19 mm, and resistant, ≤ 15 mm for clindamycin; and susceptible, ≥ 18 mm, and resistant, ≤ 13 mm for azithromycin.

Induction of resistance. To investigate the inducibility of clindamycin resistance in the presence of erythromycin, selected erythromycin-resistant isolates appearing susceptible to clindamycin were inoculated into tubes containing 2 ml of Mueller-Hinton broth supplemented with 5% lysed horse blood and 2 μg of erythromycin per ml. The tubes were incubated at 35°C in CO_2 for 4 h and then tested for induction of resistance by spot inoculation of 10 μl onto a set of three blood agar plates containing erythromycin (1 $\mu\text{g/ml}$), clindamycin (0.5 $\mu\text{g/ml}$), and erythromycin (2 $\mu\text{g/ml}$) plus clindamycin (0.5 $\mu\text{g/ml}$). The plates were incubated for 24 h in 5% CO_2 and then inspected for growth.

RESULTS

Agar dilution. Penicillin susceptibility testing was performed on the 124 pneumococcal strains and showed that 28 strains were susceptible (MICs, 0.015 to 0.06 $\mu\text{g/ml}$), 18 were intermediate (MICs, 0.12 to 0.5 $\mu\text{g/ml}$), and 78 were resistant (MICs, 2 to 8 $\mu\text{g/ml}$). Erythromycin-resistant strains were distributed in all groups, with 16 being in the penicillin-susceptible group, 18 in the penicillin-intermediate group, and 58 in the penicillin-resistant group (Table 1). Distribution of MICs of erythromycin and clindamycin with incubation in air and CO_2 for 24 h is shown in Fig. 1 and 2.

For erythromycin and clindamycin, agar dilution results correlated well with E-test and disk diffusion results (Tables 2 and 3). All erythromycin-resistant strains were classified as resistant to erythromycin by agar dilution with incubation in either air or CO_2 . Of 92 strains classified as erythromycin resistant, erythromycin MICs were ≥ 32 $\mu\text{g/ml}$ for 51 strains with incubation in air and for 70 strains with incubation in CO_2 (Fig. 1). All erythromycin-susceptible strains were classified as susceptible to erythromycin and clindamycin regardless of whether

TABLE 1. Distribution of *S. pneumoniae* strains according to their susceptibilities to erythromycin, clindamycin, and penicillin

Penicillin category	No. of strains with the following resistance phenotype:		
	Erythromycin susceptible, clindamycin susceptible	Erythromycin resistant, clindamycin susceptible	Erythromycin resistant, clindamycin resistant
Susceptible	11	6	10
Intermediate	1	7	11
Resistant	20	21	37
All strains	32	34	58

they were tested in ambient air or CO_2 (Fig. 1). The MIC at which 90% of strains are inhibited (MIC_{90}) for erythromycin in this susceptible group using incubation in air for 24 h was ≤ 0.03 $\mu\text{g/ml}$ and was 0.06 $\mu\text{g/ml}$ with incubation in CO_2 . Among the 58 clindamycin-resistant strains, 2 strains were incorrectly classified as susceptible by agar dilution using incubation in air (MICs of 0.03 and 0.06 $\mu\text{g/ml}$), and this was also true for 1 strain with incubation in CO_2 (MIC of 0.25 $\mu\text{g/ml}$) (Fig. 2). The MIC_{90} of clindamycin was 0.06 $\mu\text{g/ml}$ with incubation in air and 0.12 $\mu\text{g/ml}$ with incubation in CO_2 (Table 3).

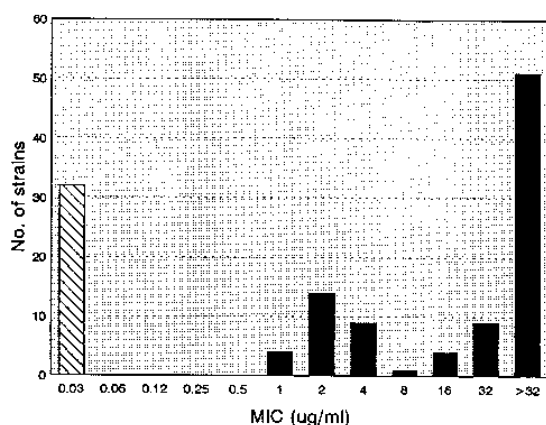
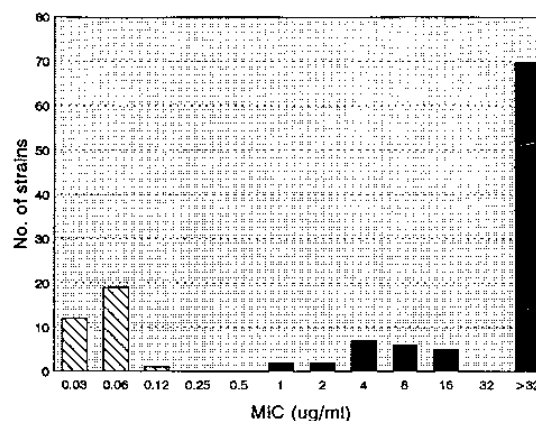
Broth microdilution. Initially, it appeared that 25 erythromycin-resistant strains had inducible clindamycin resistance when tested by microdilution in ambient air, as these strains showed trailing endpoints in the clindamycin wells at 24 h and MICs were higher at 48 h. However, when these isolates were tested for susceptibility to clindamycin by the other three methods, such isolates were shown to be highly clindamycin resistant after 24 h of incubation (Tables 2 and 3). Furthermore, although these isolates did not require CO_2 for growth (as evidenced by good growth in the control wells), all but one isolate were detected as resistant to clindamycin in 24 h by broth microdilution using CO_2 incubation, and that isolate became resistant at 48 h in CO_2 . For clindamycin, therefore, broth microdilution with incubation in air produced 33 very major errors at 24 h and 13 at 48 h, with the MIC_{50} for clindamycin-resistant strains increasing from 1 $\mu\text{g/ml}$ at 24 h to ≥ 32 $\mu\text{g/ml}$ at 48 h.

The effect of CO_2 incubation on susceptibility testing of erythromycin by broth microdilution was not as marked as it was for clindamycin (Fig. 1). All erythromycin-resistant strains were classified as resistant by this method using incubation in 5% CO_2 , while one was susceptible and three were intermediate at 24 h in air; all were correctly classified at 48 h in both atmospheres.

Disk diffusion. Most of the resistant isolates showed no inhibition zones around the disks or had inhibition zones far smaller than the breakpoints, while susceptible strains had zones well above the breakpoints at 24 h (Fig. 3). For erythromycin- and clindamycin-susceptible isolates, the zones of inhibition were ≥ 26 and ≥ 23 mm, respectively. One isolate was classified as clindamycin resistant by disk diffusion and susceptible by agar dilution and E-test at 24 h (Table 2). The clindamycin MIC at 24 h for this isolate was 0.25 $\mu\text{g/ml}$ (the upper limit of susceptible), but there was no inhibition of growth around the clindamycin disk. This strain became resistant at 48 h by agar dilution and E-test. Disk diffusion therefore clearly differentiated erythromycin- and clindamycin-susceptible isolates from resistant isolates.

To evaluate cross-resistance with other members of the macrolide-azalide group in *S. pneumoniae*, clarithromycin and azithromycin were also tested by disk diffusion. All pneumo-

a. Agar dilution incubated aerobically

b. Agar dilution incubated in CO₂

c. Broth microdilution incubated aerobically

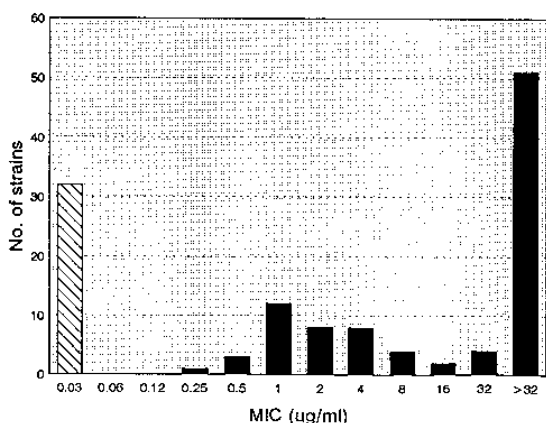
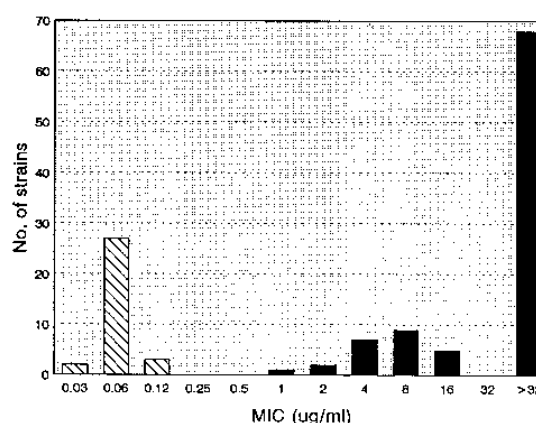
d. Broth microdilution incubated in CO₂

FIG. 1. Erythromycin MIC distribution in air and CO₂ by agar dilution and broth microdilution after incubation for 24 h for susceptible strains (hatched bars) and resistant strains (solid bars).

coccal isolates that were resistant to erythromycin were also resistant to clarithromycin and azithromycin (Fig. 3). For clarithromycin, zones of inhibition were ≤ 16 mm for resistant isolates and zones were ≥ 26 mm for susceptible isolates. Comparable zones for azithromycin were ≤ 12 mm for resistant strains and ≥ 21 mm for susceptible strains.

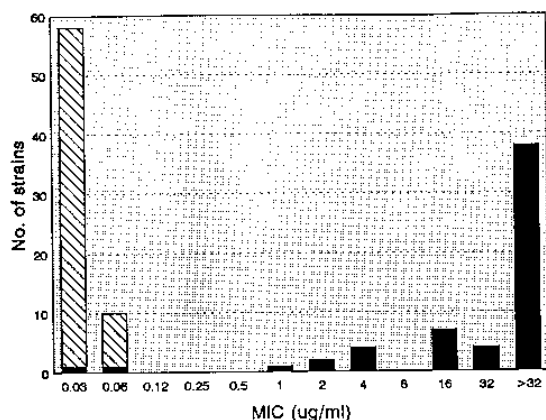
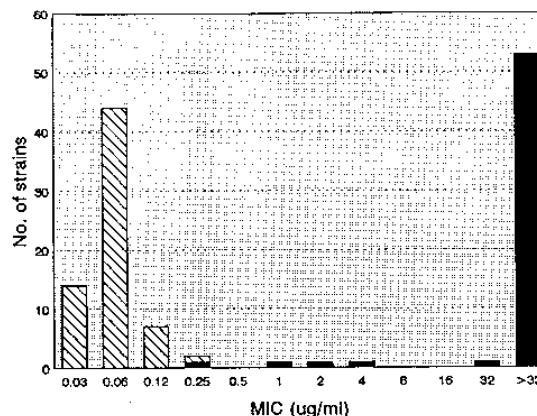
E-test. E-test results correlated well with the other methods, although MICs for susceptible strains tended to be one or two dilutions higher than those observed by agar and broth dilution methods (Table 3). The erythromycin MIC₅₀ and MIC₉₀ for erythromycin-susceptible strains were both 0.12 $\mu\text{g/ml}$. The clindamycin MIC₅₀ for clindamycin-susceptible strains was 0.12 and the MIC₉₀ was 0.25 $\mu\text{g/ml}$. MICs of both antimicrobial agents were ≥ 256 $\mu\text{g/ml}$ for most erythromycin- and clindamycin-resistant isolates by this method.

Induction of resistance. Clindamycin resistance could not be induced by preincubation with erythromycin (as described in Materials and Methods) in pneumococcal strains that were resistant to erythromycin but susceptible to clindamycin, suggesting that these strains were truly susceptible to clindamycin.

pH effect of incubation in CO₂. The effect of erythromycin and clindamycin on the pH of the media used and the effect of incubation in air versus CO₂ were determined. The pH of the

agar and broth media used was between 7.2 and 7.4 before incubation and addition of antimicrobial agents. The pH of the agar medium with the highest concentration of erythromycin tested (64 $\mu\text{g/ml}$) was 7.3 on preparation, 7.5 after incubation of uninoculated medium for 24 h in air, and 7.3 after 48 h; corresponding values after incubation in CO₂ were 7.1 and 7.0. For clindamycin, pH of the medium was 7.3 on preparation, 7.5 and 7.4 after incubation in air for 24 and 48 h, respectively, and 7.1 and 7.0 after incubation in CO₂ for 24 and 48 h, respectively. Similar pH changes were found after incubation of the broth media containing 32 μg of these agents per ml. pH of the media therefore was slightly higher than that for drug-free media at the highest concentrations of the agents, which are both alkaline and known to be less active as the pH is lowered. With incubation in air the pH rose slightly in 24 h but was close to the initial values after 48 h. However, with incubation in CO₂ pH fell to 7.1 after 24 h and 7.0 after 48 h, which could result in higher MICs due to lower activity of the agents at lower pHs. This effect was noted for susceptible strains, when modal MICs rose from ≤ 0.03 $\mu\text{g/ml}$ in air to 0.06 $\mu\text{g/ml}$ in CO₂ in 24 h for both agents. However, MICs for many resistant strains were considerably higher after incubation for 24 h in CO₂ or in air for 48 h than after incubation in air for 24 h (Fig.

a. Agar dilution incubated aerobically

b. Agar dilution incubated in CO₂

c. Broth microdilution incubated aerobically

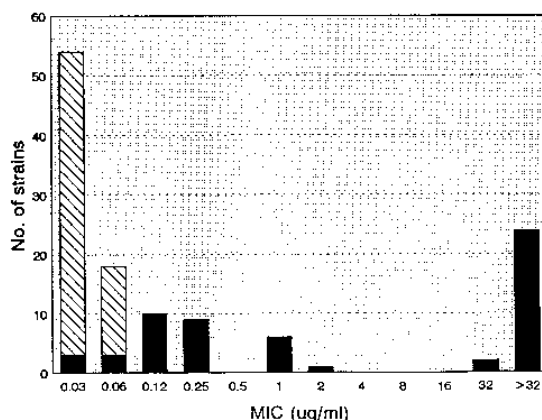
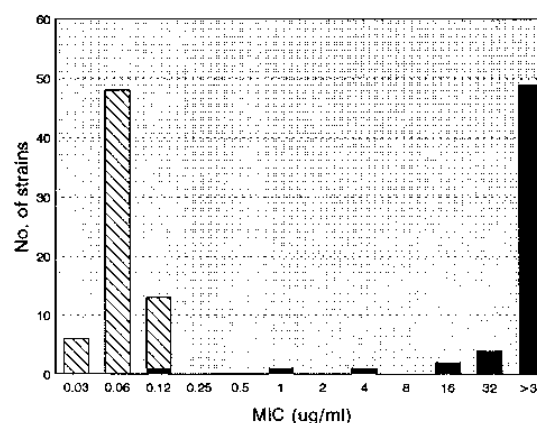
d. Broth microdilution incubated in CO₂

FIG. 2. Clindamycin MIC distribution in air and CO₂ by agar dilution and broth microdilution after incubation for 24 h for susceptible strains (hatched bars) and resistant strains (solid bars).

1 and 2), which indicates that expression of resistance has a more profound effect on MICs than the pH changes.

DISCUSSION

Erythromycin resistance among *S. pneumoniae* isolates has increased in the United States from approximately 0.2% in the

TABLE 2. Number of strains of *S. pneumoniae* susceptible to erythromycin and clindamycin according to test method, length of incubation, and incubation atmosphere^a

Antibiotic	No. of strains susceptible by the following test method after incubation for 24/48 h ^b :			
	Agar dilution MIC (CO ₂)	Broth microdilution MIC (air)	E-test (CO ₂)	Disk diffusion (CO ₂)
Erythromycin	32/32	33 ^c /32	32/32	32/32
Clindamycin	67/66	91/71	67/66	66/66

^a Of the 124 strains tested, 32 were susceptible to erythromycin and 66 were susceptible to clindamycin. Susceptible breakpoints for erythromycin were ≤ 0.25 $\mu\text{g/ml}$ for MICs and ≥ 21 mm for disk diffusion; corresponding breakpoints for clindamycin were ≤ 0.25 $\mu\text{g/ml}$ and ≥ 19 mm, respectively.

^b The incubation atmosphere is in parentheses.

^c MICs were in the intermediate range for three additional resistant strains.

late 1980s to 5% (9) and 15% (5) in some areas today. Several European countries have also reported an increase in erythromycin resistance, especially in France, where the resistance rate increased from 6% in 1970 to 25% in 1985 (2). In Spain, the macrolide resistance rate doubled in only 2 years, from 4 to 8.4% (2). Other countries, such as South Africa (9), have also reported an increased prevalence of erythromycin-resistant strains. In Hong Kong, the prevalence increased from 0% in 1983 to almost 40% in 1995 (8). An increase in erythromycin resistance has been also reported among group A streptococcal strains (6, 17), and this seems to correlate with increased clinical use of this agent in some areas (6), which could similarly play a role in the increase in macrolide resistance among pneumococci.

All erythromycin-resistant pneumococci reported to date have been cross-resistant to the macrolide and azalide agents, including clarithromycin and azithromycin (7, 10). Most of these erythromycin-resistant strains were also resistant to clindamycin, particularly those reported from South Africa and Europe. However, more recent reports described the detection of erythromycin-resistant pneumococcal isolates in the United States that were susceptible to clindamycin (3, 15). Similar findings have recently also been reported for group A strepto-

TABLE 3. MIC₅₀ and MIC₉₀ of erythromycin and clindamycin according to test method and atmosphere after incubation for 24 h

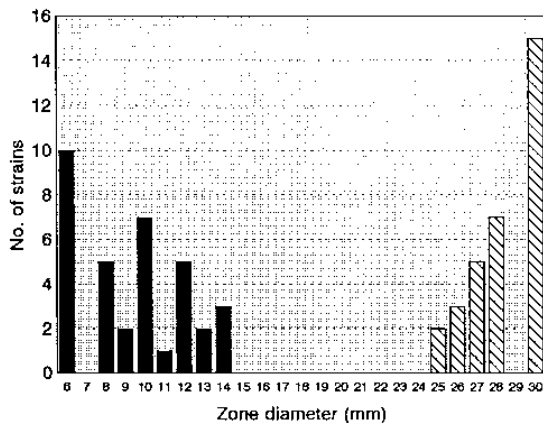
Group (no. of strains)	Result (µg/ml) in the following atmosphere by the following method:									
	Air					CO ₂				
	Microdilution		Agar dilution		Microdilution		Agar dilution		E-test	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Erythromycin susceptible (32)	≤0.03	≤0.03	≤0.03	≤0.03	0.06	0.06	0.06	0.06	0.12	0.12
Erythromycin resistant (92)	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
Clindamycin susceptible (66)	≤0.03	0.06	≤0.03	0.06	0.06	0.12	0.06	0.12	0.12	0.25
Clindamycin resistant (58)	1	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32

cocci in Finland (17). Our study has confirmed the presence of erythromycin-resistant clindamycin-susceptible strains in the United States and also detected this resistance pattern in strains from Japan, Korea, and Eastern Europe. The mechanism of resistance has recently been determined to be a macrolide efflux system which is functionally and genetically distinct from the macrolide efflux system found in coagulase-negative staphylococci (18).

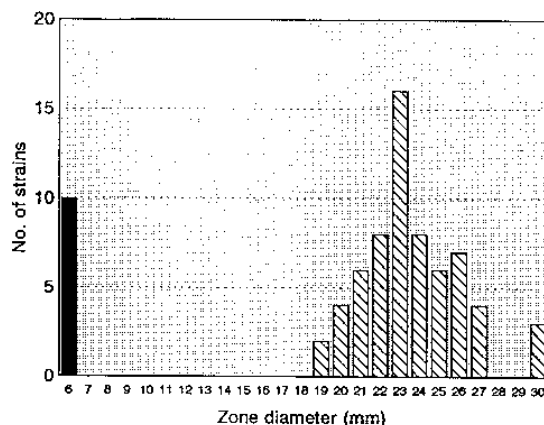
Incubation of macrolides in CO₂ has been reported to raise

MICs due to acidification of the medium by CO₂ resulting in decreased macrolide activity (20). While this effect was seen in our study, MICs for susceptible strains remained in the susceptible range, while expression of resistance in resistant strains became much more marked, resulting in clear separation of susceptible and resistant populations. As this effect was seen with extension of incubation time in ambient air, the reason for this effect is postulated to be due to more rapid growth of strains in the presence of CO₂, with earlier expres-

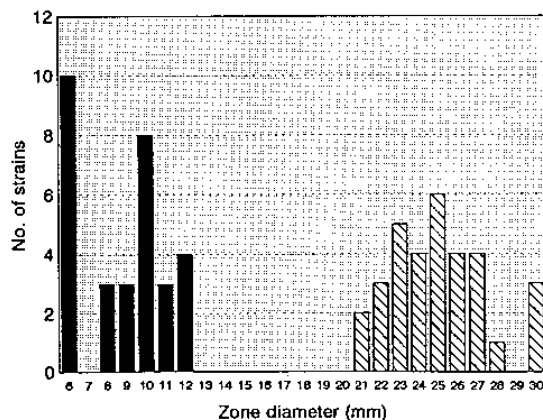
a. Erythromycin



b. Clindamycin



c. Azithromycin



d. Clarithromycin

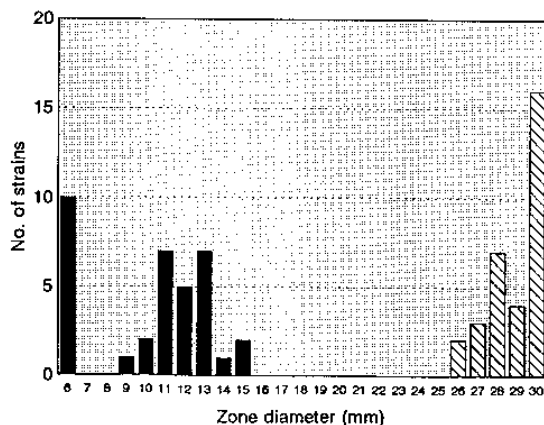


FIG. 3. Distribution of zone diameters at 24 h by disk diffusion with susceptible strains (hatched bars) and resistant strains (solid bars). For clarity only 10 strains for which zone diameters were 6 mm are shown; actual numbers of strains are erythromycin, 67; clindamycin, 58; azithromycin, 71; and clarithromycin, 67.

sion or induction of macrolide resistance and not due to the rather small changes in pH noted.

In our study, many strains of erythromycin-resistant pneumococci appeared susceptible to clindamycin at 24 h by microdilution when incubated in ambient air but were resistant by the other three other methods which include incubation in CO₂ and by microdilution when incubated in CO₂. The effect of CO₂ incubation on susceptibility testing of erythromycin was not as marked as it was for clindamycin, and most erythromycin-resistant isolates could be detected regardless of whether they were tested in ambient air or CO₂. These findings are in agreement with those of a previous article (10). However, the NCCLS microdilution method, which specifies incubation in ambient air for 20 to 24 h, produced significant very major errors (43%; 25 of 58 strains) with clindamycin. Modification by incubation in CO₂ or extension of incubation time to 48 h corrected these errors. Disk diffusion, however, was the simplest and most convenient test method, with the same accuracy as or accuracy superior to agar dilution.

This study has documented problems associated with the NCCLS microdilution method for susceptibility testing of pneumococci, while showing the reliability of other methods. In addition, macrolide breakpoints recommended by the NCCLS should be modified to include intermediate strains as resistant, as such strains express higher levels of resistance with further incubation or with incubation in CO₂.

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