Prevalence of Antimicrobial Resistance in *Haemophilus influenzae* and *Streptococcus pneumoniae*: Comparison of Clinical Isolates of Japan and The Philippines

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Abstract: For clinical isolates of *Haemophilus influenzae* and *Streptococcus pneumoniae* in Japan (356 and 179 strains, respectively) and in the Philippines (98 and 59 strains, respectively), minimum inhibitory concentrations (MICs) of ampicillin, cefazolin, cefotiam, ceftizoxim, ofloxacin, erythromycin, and minocycline were examined. The rates of β-lactamase producing *H. influenzae* were 17.7% (63/356) in Japan and 2.0% (2/98) in the Philippines, and all of these strains were ampicillin MICs $\geq$1.56 ugml^{-1}. In addition, 5 strains in Japan that lacked β-lactamase activity were also less susceptible to ampicillin. Among the antimicrobials tested, ceftizoxim was the most active against *H. influenzae* in both countries (MICs $\leq$0.2 ugml^{-1}). Five strains of *S. pneumoniae* in Japan were relatively resistant to ampicillin (MIC=0.1 ugml^{-1}), whereas there were no such strains among isolates in the Philippines. Forty strains (22.3%) and 108 strains (60.3%) among *S. pneumoniae* in Japan exhibited erythromycin MICs $\geq$0.2 ugml^{-1} and minocycline MICs $\geq$1.56 ugml^{-1}, respectively. In contrast, all isolates in the Philippines were erythromycin MICs $\leq$0.05 ugml^{-1} and minocycline MICs $\leq$0.39 ugml^{-1}. Present study indicates that *H. influenzae* and *S. pneumoniae* in the Philippines remained still susceptible to the antimicrobials tested except for 2 β-lactamase-positive, ampicillin-resistant *H. influenzae,* whereas ampicillin-resistant *H. influenzae* mediated by β-lactamase or non-β-lactamase mechanisms and ampicillin-, erythromycin- or minocycline-resistant *S. pneumoniae* were included among isolates in Japan.

Key words: Antimicrobial resistance, *H. influenzae, S. pneumoniae,* Japan, The Philippines

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INTRODUCTION

Haemophilus influenzae (Everett et al., 1977; McGowan et al., 1974) and *Streptococcus pneumoniae* (Davies and Kumaratne 1988; Gillespie 1989) were important bacterial pathogens causing systemic infections, such as sepsis and meningitis, in addition to respiratory tract infections. Recently, increase of prevalence of resistance in *H. influenzae* (Istre et al., 1984; Kayser et al., 1990) and *S. pneumoniae* (Baquero et al., 1991; Ridgway et al., 1991; Spika et al., 1991) have been clinically becoming problems. It is important to monitor for the prevalence of antimicrobial resistance in each country for treating invasive, life-threatening infections caused by these organisms. Surveys of the prevalence of resistance in *H. influenzae* (Kayser et al., 1990; Doern et al., 1986; Doern et al., 1988; Powell et al., 1987) and *S. pneumoniae* (Baquero et al., 1991; Spika et al., 1991) have been carried out in several countries.

In this study, antimicrobial susceptibility of clinical isolates of *H. influenzae* and *S. pneumoniae* in Japan and in the Philippines was investigated, and the prevalence of resistance of these organisms was compared. The rate of β-lactamase producing strains in *H. influenzae* and the correlation of resistance between erythromycin (EM) and minocycline (MINO) in *S. pneumoniae* were also examined.

MATERIALS AND METHODS

Clinical isolates. For isolates in Japan, 356 strains of *H. influenzae* and 179 strains of *S. pneumoniae* collected between December 1986 and November 1987 at Nagasaki University Hospital, Nagasaki, Japan, were used. For isolates in the Philippines, 98 strains of *H. influenzae* and 59 strains of *S. pneumoniae* collected between December 1984 and November 1985 at Research Institute for Tropical Medicine, Manila, the Philippines, were used. All isolates were recovered from different patients. The isolates in the Philippines were sent to Nagasaki University Hospital. After confirmation of identity, antimicrobial susceptibility testing for isolates in both countries was performed at one time at Nagasaki University Hospital.

Antibiotics. The following 7 antimicrobial agents were obtained from the indicated sources: ampicillin (ABPC), Meiji Seika Kaisha, Ltd., Tokyo, Japan; cefazolin (CEZ), Fujisawa Pharmaceutical Co., Ltd., Tokyo, Japan; cefotiam (CTM), Takeda Chemical Industries Ltd., Osaka, Japan; ceftizoxime (CZX), Fujisawa; ofloxacin (OFLX), Daiichi Pharm. Co., Ltd., Tokyo, Japan; EM, Dainippon Pharm. Co., Ltd., Osaka, Japan; MINO, Lederle (Japan) Ltd., Tokyo, Japan.

Antimicrobial susceptibility testing. Minimum inhibitory concentrations (MICs) of antibiotics against *H. influenzae* and *S. pneumoniae* were determined by broth microdilution method using serial twofold dilutions of an individual antibiotic (Sahm and Washington II 1991). Test medium consisted of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.), 50mg of nicotinamide adenine dinucleotide (Sigma Chemical Co., St. Louis, Mo.) per liter, 50 ml of heat-inactivated fetal calf serum (Nakarai Chemical Co., Ltd., Tokyo, Japan) per liter,

30ml of lysed horse blood (Nissei kagaku Co., Ltd., Tokyo, Japan) per liter, 3g of yeast extract (Oxoid, Ltd., Basingstoke, Hampshire, UK) per liter, 25mg of magnesium per liter, and 50mg of calcium per liter. The 7 antimicrobial agents were examined against all study isolates at concentrations ranging from 0.025 to 50 $ugml^{-1}$. Bacteria were inoculated to obtain a final concentration of approximately 10^5 $CFUml^{-1}$, and test plates were incubated for 20 to 24h at 35C in ambient air. Then, wells were examined macroscopically for evidence of growth. The MIC was defined as the lowest concentration of antimicrobial agent tested which inhibited visible growth of bacteria. Strains of *H. influenzae* ATCC 35056, Pseudomonas aeruginosa ATCC 27853, *Escherichia* coli ATCC 25922, and *Staphylococcus* aureus ATCC 29213 for which the MICs of the antimicrobial agents were known, were used as control of antimicrobial susceptibility testing.

Detection of β-lactamase. β-lactamase production in *H. influenzae* was determined by incubating suspensions of each strain with chromogenic cephalosporin (nitrocefin) at room temperature for 10 min and visually assessing color development (O'Callaghan et al., 1972).

RESULTS

MICs of ABPC, CEZ, CTM, and CZX against *H. influenzae*. The in vitro activities of 4 β-lactam antibiotics against 356 strains in Japan and 98 strains in the Philippines were shown in Table 1. MICs for 50% of strains (MIC50s) of ABPC, CEZ, CTM, and CZX for strains in Japan were respectively 0.39, 6.25, 0.78, and $\leq$0.025 $ugml^{-1}$, whereas MIC50s for strains in the Philippines were 0.2, 3.13, 0.78, and $\leq$0.025 $ugml^{-1}$, respectively. Concerning the distribution of MICs of ABPC, there was a distinct difference between Japan and the Philippines. Only 2 strains in the Philippines (2.0%) were resistant to ABPC (MICs of 12.5 $ugml^{-1}$ and 25 $ugml^{-1}$), whereas 50 strains in Japan (14.0%) exhibited ABPC MICs $\geq$6.25 $ugml^{-1}$. Among β-lactam antimicrobial agents tested, CZX was the most active against *H. influenzae* in both countries. All 98 strains in the Philippines and 343 strains in Japan (96.3%) were inhibited by CZX at a concentration of 0.025 $ugml^{-1}$, but remaining 13 strains isolated in Japan exhibited resistance to CZX at 0.05 $ugml^{-1}$ (8 strains), 0.1 $ugml^{-1}$ (4 strains), and 0.2 $ugml^{-1}$ (1 strain) of CZX, respectively.

MICs of ABPC for beta-lactamase-positive and -negative strains of *H. influenzae*. The rate of β-lactamase production among strains in Japan was 17.7% (63/356 strains). In contrast, 2.0% of strains (2/98 strains) in the Philippines produced β-lactamase (Table 2). All of these β-lactamase producing strains were ABPC MICs $\geq$1.56 $ugml^{-1}$. In addition, 5 strains (1.4%) in Japan that lacked β-lactamase activity showed ABPC MICs $\geq$1.56 $ugml^{-1}$. These 5 strains were also less susceptible to CZX; CZX MICs of 0.2 $ugml^{-1}$ (1 strain), 0.1 $ugml^{-1}$ (3 strains), and $\leq$0.025 $ugml^{-1}$ (1 strain). However, all 96 strains in the Philippines that lacked β-lactamase activity were ABPC MICs $\leq$0.39 $ugml^{-1}$.

MICs of OFLX, EM, and MINO against *H. influenzae*. The in vitro activities of 3 other classes of antimicrobial agents against *H. influenzae* were shown in Table 3. In MIC50s of OFLX, EM, and MINO, there were no differences between strains in Japan and in the

Table 1. MICs of ABPC, CEZ, CTX, and CZX for *H. influenzae* in Japan (356 strains) and in the Philippines (98 strains)

Country	Antimicrobial agent	No. of strains for which MIC (μgml^{-1}) was as follows [a]:												
		≤0.025	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	>50
Japan	ABPC			1	110	175	2	6	12	10	17	18	5	
	CEZ			1	1			20	139	174	19	1	1	
	CTM			1	3	58	233	51	9	1				
	CZX	343	8	4	1									
The Philippines	ABPC			25	70	1					1	1		
	CEZ						1	14	37	36	10			
	CTM				1	39	55	3						
	CZX	98												

[a]MIC50 of each antibiotic was indicated by underline.

Table 2. MICs of ABPC for β-lactamase-positive and -negative strains of *H. influenzae* in Japan (356 strains) and in the Philippines (98 strains)

Country	β-lactamase	No. of strains for which MIC (μgml^{-1}) was as follows:									
		0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50
Japan	Positive					3	10	10	17	18	5
	Negative	1	110	175	2	3	2				
The Philippines	Positive								1	1	
	Negative	25	70	1							

Table 3. MICs of OFLX, EM, and MINO for *H. influenzae* in Japan (356 strains) and in the Philippines (98 strains)

Country	Antimicrobial agent	No. of strains for which MIC (μgml^{-1}) was as follows [a]:												
		≤0.025	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	>50
Japan	OFLX	304	39	4	6	1								
	EM				4	2	15	74	205	56				
	MINO		12	150	139	31	17	5		1	1			
The Philippines	OFLX	73	24	1										
	EM						1	15	57	25				
	MINO			1	63	33	1							

[a]MIC50 of each antibiotic was indicated by underline.

Philippines ($\leq$0.025, 3.13, and 0.2 ugml^{-1} respectively). However, 2 strains in Japan were fully resistant to MINO (MICs of 6.25 ugml^{-1} and 12.5 ugml^{-1}), although all 98 strains in the philippines were MINO MICs $\leq$0.78 ugml^{-1}.

MICs of ABPC, CEZ, CTM, and CZX against *S. pneumoniae*. The in vitro activities of 4 β-lactam antibiotics against 179 strains in Japan and 59 strains in the Philippines were shown in Table 4. In MIC50s of ABPC, CEZ, CTM, and CZX, there were no differences between strains in Japan and in the Philippines ($\leq$0.025, 0.1, 0.1, and 0.05 ugml^{-1}, respectively). Among these β-lactam antimicrobial agents, ABPC was the most active against *S. pneumoniae* isolated in both countries. However, 5 strains in Japan (2.8%) were relatively resistant to ABPC (MIC=0.1 ugml^{-1}).

MICs of OFLX, EM, and MINO against *S. pneumoniae*. The in vitro activities of 3 other classes of antimicrobial agents were shown in Table 5. MIC50s of OFLX, EM, and

Table 4. MICs of ABPC, CEZ, CTM, and CZX for *S. pneumoniae* in Japan (179 strains) and in the Philippines (59 strains)

Country	Antimicrobial agent	No. of strains for which MIC (μgml^{-1}) was as follows [a]:												
		$\leq$0.025	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	>50
Japan	ABPC	154	20	5										
	CEZ		30	128	13	7	1							
	CTM	1	14	90	59	15								
	CZX	17	114	27	8	9	2	2						
The Philippines	ABPC	58	1											
	CEZ		19	40										
	CTM		1	44	14									
	CZX	20	38	1										

[a]MIC50 of each antibiotic was indicated by underline.

Table 5. MICs of OFLX, EM, and MINO for *S. pneumoniae* in Japan (179 strains) and in the Philippines (59 strains)

Country	Antimicrobial agent	No. of strains for which MIC (μgml^{-1}) was as follows [a]:												
		$\leq$0.025	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	>50
Japan	OFLX					1	8	146	22	1	1			
	EM	20	109	10	1	1	4	11	8	1			1	13
	MINO	16	39	1	3	5	7	4	11	30	38	24	1	
The Philippines	OFLX						1	39	19					
	EM	13	46											
	MINO			17	40	2								

[a]MIC50 of each antibiotic was indicated by underline.

MINO for strains in Japan were respectively 1.56, 0.05, and 6.25 ugml^{-1}, whereas MIC50s for strains in the Philippines were 1.56, 0.05, and 0.2 ugml^{-1}, respectively. Among isolates in Japan, 40 strains (22.3%) were EM MICs $\geq$0.2 ugml^{-1} and 108 strains (60.3%) were MINO MICs $\geq$1.56 ugml^{-1}. However, all isolates in the Philippines were EM MICs $\leq$0.05 ugml^{-1} and MINO MICs $\leq$0.39 ugml^{-1}.

Correlation of MICs between MINO and EM for *S. pneumoniae*. Strains of *S. pneumoniae* were divided into 4 groups (A–D) by MINO MICs $<$1.56 or $\geq$1.56 ugml^{-1} and EM MICs $<$0.2 or $\geq$0.2 ugml^{-1} as shown in Figure 1 (group A, MINO MICs $<$1.56 ugml^{-1} and EM MICs $<$0.2 ugml^{-1}; B, MINO MICs $\geq$1.56 ugml^{-1} and EM MICs $<$0.2 ugml^{-1}; C, MINO MICs $\geq$1.56 ugml^{-1} and EM MICs $\geq$0.2 ugml^{-1}; D, MINO MICs $<$1.56 ugml^{-1} and EM MICs $\geq$0.2 ugml^{-1}). All isolates in the Philippines belonged to group A. In contrast, the strains in Japan were more variable. Among 108 strains in Japan (MINO MICs $\geq$1.56 ugml^{-1}), 38 strains (35.2%, 38/108 strains) were EM MICs $\geq$0.2 ugml^{-1} (group C), whereas among 71 strains (MINO MICs $<$1.56 ugml^{-1}), only 2 strains (2.8%, 2/71 strains) showed EM MICs $\geq$0.2 ugml^{-1} (group D).

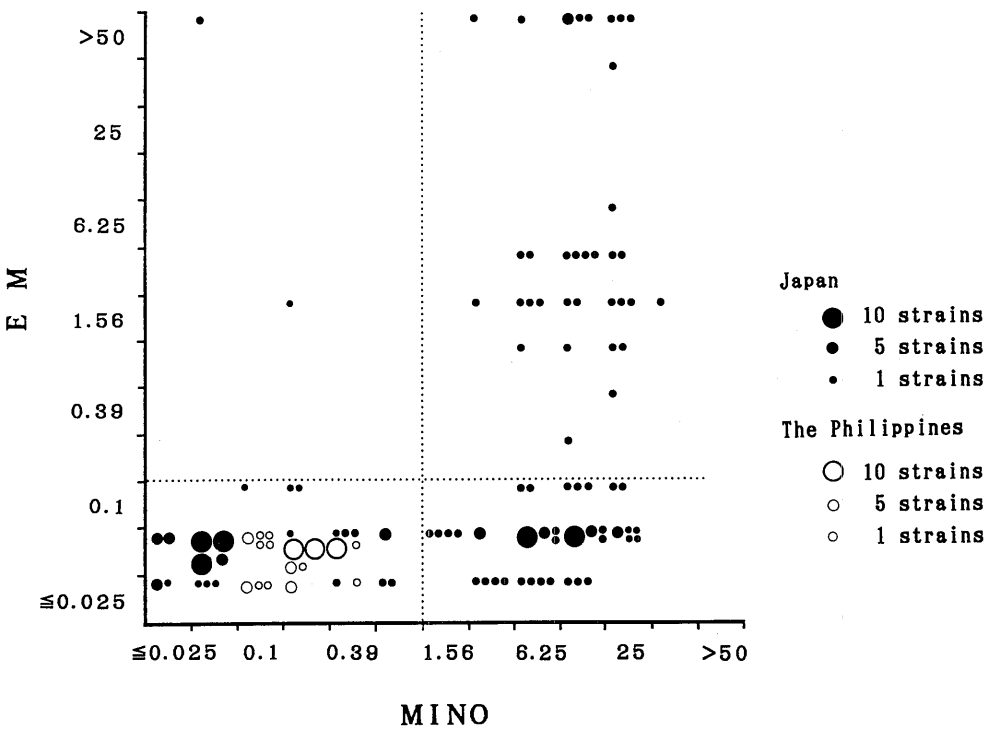

Fig. 1. **Correlation of MICs of MINO and EM for *S. pneumoniae*.** Each strain of S. pneumoniae in Japan (179 strains) and in the Philippines (59 strains) was plotted by MIC values (ugml^{-1}) of MINO and EM.

Discussion

The rates of β-lactamase producing strains of *H. influenzae* in Japan and in the Philippines were 17.7% (63/356 strains) and 2.0% (2/98 strains), respectively. The vast majority of β-lactamase produced by *H. influenzae* has been known to be TEM-type β-lactamase (Heffron et al., 1975) and is mediated by genes located on a conjugative R-plasmid (Elwell et al., 1975; Sykes et al., 1975). From the first report of β-lactamase producing *H. influenzae* in 1974 (Khan et al., 1974) series of surveillances in prevalence of β-lactamase-positive strains have been carried out in several countries. In nationwide studies in the United States, the rates of β-lactamase producing strains of *H. influenzae* in 1984 (Doern et al., 1986) and in 1986 (Doern et al., 1988) were 15.2% and 20.0%, respectively. European collaborative studies have shown that 10.9% in 1986 (Machka et al., 1988) and 9.1% in 1988/89 (Kayser et al., 1990) produced β-lactamase. In the present study, the prevalence of β-lactamase producing strains in the Philippines remained still at low levels compared with the rates in the United States and Europe, although the prevalence in Japan was similar to that seen in the United States performed in 1986.

The problem of ABPC resistance in *H. influenzae* is complicated by descriptions of clinical isolates that are resistant to ABPC by mechanisms other than the production of β-lactamase (Mendelman et al., 1984; Offit et al., 1982). Parr and Bryan (1984) have reported that alteration in the binding capacity of penicillin-binding proteins (PBPs) of *H. influenzae* was correlated with the β-lactam resistance of this organism. Among *H. influenzae* in Japan that lacked β-lactamase production, 5 strains (1.4%) showed ABPC MICs $\geq$1.56 ugml^{-1}. These 5 strains were also less susceptible to CZX. On the other hand, all isolates in the Philippines that lacked β-lactamase activity were susceptible to ABPC, which were inhibited by ABPC concentration of 0.39 ugml^{-1}. Recent increasing use of new β-lactams, most of which are stable in the presence of β-lactamase of *H. influenzae,* is likely to increase selective pressure on ABPC-resistant strains mediated by such non-β-lactamase mechanisms.

Five strains of *S. pneumoniae* in Japan were relatively resistant to ABPC (MIC=0.1 ugml^{-1}), whereas growth of all isolates in the Philippines were inhibited by ABPC concentration of 0.05 ugml^{-1}. From the report of *S. pneumoniae* with greater resistance to penicillin (MIC=4 ugml^{-1}) in 1977 (Appelbaum et al., 1977), several surveillances for resistant *S. pneumoniae* have been carried out. Recent study in the United States has reported that 5% of isolates were ABPC MICs $\geq$0.1 ugml^{-1} (Spika et al., 1991). Other report in Europe has shown that over 25% of isolates in Spain, Hungary, and Poland were penicillin MICs $>$0.1 ugml^{-1} (Baquero et al., 1991). In Spain, a close relationship has been found between the yearly rate of aminopenicillin consumption and penicillin resistance (Baquero et al., 1991). The development of resistance to penicillin by *S. pneumoniae* has been also reported to be associated with changes in the affinity and molecular size of PBPs (Handwerger and Tomasz 1986; Jabes et al., 1989).

All isolates of *S. pneumoniae* in the Philippines were EM MICs $\leq$0.05 ugml^{-1} and MINO MICs $\leq$0.39 ugml^{-1}. In contrast, among 179 strains of *S. pneumoniae* in Japan, 40

strains (22.3%) were EM MICs $\geq$0.2 ugml^{-1} and 108 strains (60.3%) were MINO MICs $\geq$1.56 ugml^{-1} (Table 5). As shown in Fig. 1, over 35% of MINO-resistant strains were simultaneously EM-resistant, whereas only 2.8% of MINO-sensitive strains were EM-resistant. These results suggest that acquisition of MINO resistance may be prerequisite to development of EM resistance, at least, for *S. pneumoniae* tested in this study. With respect to the mechanisms of development of EM and MINO resistance in *S. pneumoniae,* a conjugative transposon carrying resistance genes for these antibiotics has been described (Courvalin and Carlier 1986). Further studies were required to elucidate the molecular mechanisms of cross-resistance between EM and MINO in *S. pneumoniae.*

In conclusion, *H. influenzae* and *S. pneumoniae* in the philippines remained still susceptible to the antimicrobials tested except for 2 strains of β-lactamase-positive, ABPC-resistant *H. influenzae,* whereas ABPC-resistant *H. influenzae* mediated by β-lactamase or non-β-lactamase mechanisms and ABPC-, MINO- or EM-resistant *S. pneumoniae* were included among isolates in Japan. Continuous surveillances to monitor for the prevalence of resistance to antimicrobials in *H. influenzae* and *S. pneumoniae* were needed.

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