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Incidence, Epidemiology, and Characteristics of Quinolone-Nonsusceptible *Streptococcus pneumoniae* in Croatia

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Among 585 *Streptococcus pneumoniae* strains isolated in 22 Croatian hospitals 21 strains (3.6%) were quinolone nonsusceptible. MICs of all quinolones were high for seven strains tested with the same serotype (23F) and mutations in *gyrA*, *parC*, and *parE*. The remaining 14 strains were more heterogeneous and had mutations only in *parC* and/or *parE*, and the MICs of quinolones were lower for these strains.

The incidence of pneumococci resistant to penicillin G and other β-lactam and non-β-lactam compounds has increased worldwide at an alarming rate, including in the United States (1, 2, 3, 8, 13). In the United States in a recent survey, 50.4% of 1,476 clinically significant pneumococcal isolates were not susceptible to penicillin and 33% were resistant to macrolides (10). The problem of drug-resistant pneumococci is compounded by the ability of resistant clones to spread from country to country and from continent to continent (14).

Several recent reports from Hong Kong (8), Canada (5), and Spain (19) have described the increasing incidence of quinolone-nonsusceptible pneumococci. A recent study from our laboratory has documented a high incidence of quinolone nonsusceptibility (8%) among pneumococcal cultures isolated from blood, tracheobronchial fluid, and sputum of adult patients in Zagreb, Croatia (15). The present study includes strains isolated from adult patients from the preceding study (15) and expands on this finding by examining the phenotype and genotype of 585 pneumococci isolated from 22 hospitals in 15 Croatian cities in 2000 and 2001.

Five hundred eighty-five Croatian pneumococcus strains were studied for their susceptibility to penicillin G, amoxicillin-clavulanic acid, erythromycin, ciprofloxacin, levofloxacin, gemifloxacin, gatifloxacin, and moxifloxacin. These clinical strains were isolated from adults in 22 hospitals from 15 Croatian cities (Fig. 1) between November 2000 and April 2001. Strains were frozen at −70 in 2% skim milk and transported by courier to Hershey Medical Center on dry ice within 2 months of isolation. All strains were from individual patients. Patient charts were examined by at least one of the Croatian authors.

The patient demographics are shown in Fig. 2. Five hundred eighty-five isolates were from 22 hospitals in 15 cities throughout Croatia. Patient ages varied between 13 and 92 years (mean, 49 years). There was a slightly higher number of isolates from patients older than 50 (53%) than from younger patients. This increase was more important for quinolone-nonsusceptible strains (59%). Most of the isolates (65%) were from male patients, and 86% of the quinolone-nonsusceptible strains were isolated from male patients. Fifty-seven percent of all isolates and 82% of quinolone-resistant strains were nosocomial. Older age and male sex are two important risk factors for pneumococcal pneumonia (12). The number of pneumococcal isolates increased with age in Croatia. This increase was more important for the infections caused by quinolone-nonsusceptible pneumococci. In Finland pneumococcal infections have been found to be more frequent among men than women (11). In Croatia very high proportions of the quinolone-nonsusceptible strains (86%) were isolated from male patients. Among patients with pneumococcal infections, older age, male sex, and hospitalization were found to be the risk factors for isolation of quinolone-nonsusceptible strains.

The main types of infection were upper respiratory tract infection (29%), community-acquired pneumonia (25%), and acute exacerbation of chronic bronchitis (16.4%) for all isolates and upper respiratory tract infection (28.6%), acute exacerbation of chronic bronchitis (14.3%), and pneumonia (14.3%) for quinolone-nonsusceptible strains. Overall isolates were from sputum (36.8%), nasopharynx (34.4%), tracheobronchial aspirates (16%), and blood (7%). Quinolone-nonsusceptible strains were from tracheobronchial aspirates (28.6%), nasopharynx (28.6%), sputum (19%), and blood (9%).

All antimicrobials were obtained from their respective manufacturers. Agar dilution was performed with Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood (6).

The MICs for 585 *S. pneumoniae* isolates are shown in Table 1. Twenty-one strains were quinolone nonsusceptible, defined as a ciprofloxacin MIC of ≥4 μg/ml. Of 585 strains, 64% were susceptible to penicillin, 22% were intermediate, and 14% were resistant. Eighteen percent of strains were resistant to macrolides. Two strains (0.3%) were resistant to penicillin, erythromycin, and ciprofloxacin; 21 strains (3.6%) were resis-

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FIG. 1. Distribution of the 585 pneumococci isolated from 22 hospitals in 15 Croatian cities in 2000 and 2001.

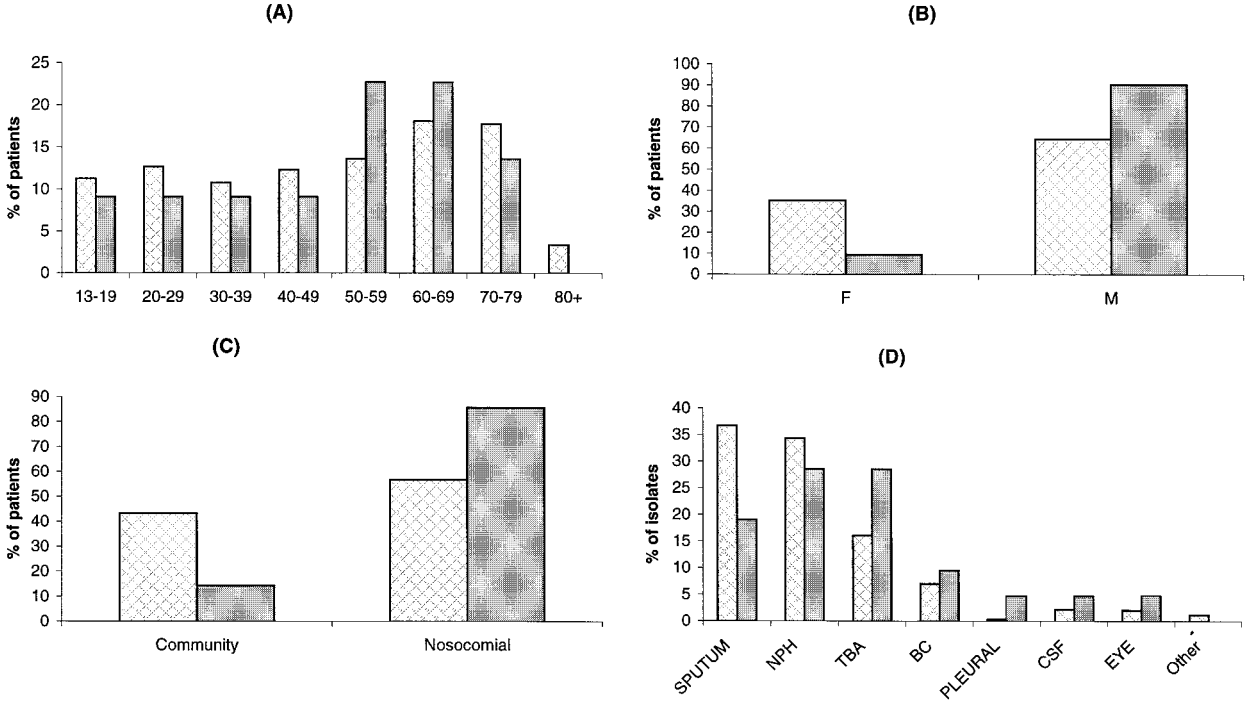

FIG. 2. Demographics of *S. pneumoniae* strains isolated in Croatia. Distribution of total strains and quinolone-resistant strains by age (A), sex (B), hospitalization (C), and isolation site (D) is shown. Total strains (585 strains) are indicated by crosshatched bars, and quinolone-nonsusceptible strains (21 strains) are shown by shaded bars, NPH, nasopharyngeal swab; TBA, tracheobronchial aspirates; BC, blood culture; CSF, cerebrospinal fluid.

TABLE 1. MICs (micrograms per milliliter) for 585 pneumococcal strains[a]

Drug and strain group	MIC range	MIC_{50}	MIC_{90}
Penicillin G			
All strains	≤0.008–>16	0.03	2
Penicillin S	≤0.008–0.06	0.03	0.03
Penicillin I	0.125–1	0.5	1
Penicillin R	2–>16	2	4
Quinolone S	≤0.008–>16	0.03	2
Quinolone N	0.008–4	2	4
Amoxicillin-clavulanic acid			
All strains	≤0.008–16	0.03	2
Penicillin S	≤0.008–0.125	0.03	0.03
Penicillin I	0.03–2	0.25	1
Penicillin R	0.125–16	2	2
Quinolone S	≤0.008–16	0.03	1
Quinolone N	0.016–4	1	2
Erythromycin			
All strains	≤0.008–>64	0.03	>64
Penicillin S	≤0.008–>64	0.03	0.06
Penicillin I	≤0.008–>64	0.03	>64
Penicillin R	≤0.008–>64	0.03	>64
Quinolone S	≤0.008–>64	0.03	>64
Quinolone N	≤0.008–>64	0.03	>64
Ciprofloxacin			
All strains	0.5–>32	1	2
Penicillin S	0.5–8	1	2
Penicillin I	0.5–4	1	2
Penicillin R	0.5–>32	1	8
Quinolone S	0.5–2	1	2
Quinolone N	4–>32	8	16
Levofloxacin			
All strains	0.5–32	1	2
Penicillin S	0.5–8	1	2
Penicillin I	1–4	1	2
Penicillin R	0.5–32	1	16
Quinolone S	0.5–2	1	2
Quinolone N	2–32	4	16
Gemifloxacin			
All strains	≤0.008–0.5	0.03	0.06
Penicillin S	≤0.008–0.125	0.03	0.06
Penicillin I	0.016–0.06	0.03	0.06
Penicillin R	0.016–0.5	0.03	0.125
Quinolone S	0.008–0.06	0.03	0.06
Quinolone N	0.03–0.5	0.06	0.125
Gatifloxacin			
All strains	0.125–8	0.25	0.5
Penicillin S	0.125–2.0	0.25	0.5
Penicillin I	0.25–1.0	0.25	0.5
Penicillin R	0.25–8.0	0.25	4
Quinolone S	0.125–0.5	0.25	0.5
Quinolone N	0.5–8	1	4
Moxifloxacin			
All strains	0.06–4	0.125	0.25
Penicillin S	0.06–0.5	0.125	0.25
Penicillin I	0.06–0.25	0.125	0.25
Penicillin R	0.125–4	0.125	2
Quinolone S	0.06–0.5	0.125	0.25
Quinolone N	0.25–4	0.5	2

[a] Of 585 strains tested, 378 were susceptible, 127 strains were intermediate, and 80 strains were resistant to penicillin and 564 strains were susceptible and 21 were nonsusceptible to quinolones. Quinolone nonsusceptibility is defined by a ciprofloxacin MIC of ≥4 μg/ml. S, susceptible; I, intermediate; R, resistant; N, nonsusceptible.

tant to penicillin and erythromycin; and 12 strains (2.1%) were resistant to penicillin and ciprofloxacin. Three hundred thirty-three strains (56.4%) were susceptible to all three antibiotics. Amoxicillin-clavulanic acid was active against 99% of total isolates and 95% of quinolone-nonsusceptible strains at a MIC of ≤2 μg/ml, the present NCCLS breakpoint (17). Twenty strains (3.4%) were levofloxacin nonsusceptible: 11 of 20 were intermediate resistant (MIC = 4 μg/ml) and 9 of 20 were resistant (MIC > 4 μg/ml) to levofloxacin.

Among quinolones tested gemifloxacin had the lowest MICs against both quinolone-susceptible and quinolone-resistant strains (MIC at which 90% of the isolates tested were inhibited [MIC_{90}] = 0.06 μg/ml), followed by moxifloxacin (MIC_{90} = 0.25 μg/ml), gatifloxacin (MIC_{90} = 0.5 μg/ml), levofloxacin (MIC_{90} = 2 μg/ml), and ciprofloxacin (MIC_{90} = 2 μg/ml) (Table 1).

To determine quinolone resistance mechanisms, quinolone resistance determinant regions in *parC*, *parE*, *gyrA*, and *gyrB* genes were amplified by PCR as described by Pan et al. (18), purified with a QIAquick PCR purification kit (Qiagen, Valencia, Calif.), and sequenced in the forward direction unless a new mutation was observed, in which case sequencing was done in both directions directly with an Applied Biosystems Model 373A DNA sequencer.

Twenty-one quinolone-nonsusceptible strains were studied further for their resistance mechanisms (Table 2), for their serotype by the capsule Quellung method (9) with commercial antisera (Statens Seruminstitut, Copenhagen, Denmark) as recommended by the manufacturer, and for their clonality by pulsed-field gel electrophoresis (16, 20). The analysis of the results indicates that quinolone-nonsusceptible strains could be separated into two groups; the first group comprised seven strains for which MICs of all the quinolones tested were relatively high and which were resistant to penicillin. All seven strains were serotype 23F and had mutations in *gyrA*, *parC*, and *parE* that led to the S81Y substitution in GyrA, the S79F substitution in ParC, and the I460V substitution in ParE, respectively. Six of these strains had the same pulsed-field gel electrophoresis type after digestion by *Sma*I. Five of these strains were isolated in the University Hospital for Lung Diseases in Zagreb (three from bronchial aspirates, one from pleural fluid, and one from blood), and one was isolated from blood in the Public Health Institute in Varazdin (Fig. 1). The strain with a different pulsed-field gel electrophoresis type was from Cakovec.

The second group was more heterogeneous. All strains were resistant to ciprofloxacin (MICs ≥ 4 μg/ml), but the MICs of the newer quinolones for these strains were lower. Of these 14 strains, 5 were serotype 11, 3 were serotype 9, 2 were serotype 14, and 3 were nontypeable. In this group eight strains were genetically related. These strains were isolated in Zagreb (4), Split (3), and Cakovec (1). This group did not have a mutation in *gyrA* or *gyrB*, and overall the MICs of quinolones were lower for these strains. They had mutations in *parC* and/or *parE*.

The prevalence of pneumococci with reduced fluoroquinolone susceptibility has been shown to be increased in Canada, Hong Kong, and Spain. Quinolone nonsusceptibility was 1.7% in Canada (5), 13.3% in Hong Kong (8), and 7.1% in Spain (19). In Hong Kong quinolone nonsusceptibility rates were higher among penicillin-resistant strains (27.3%) (8). Results

TABLE 2. Demographics of quinolone-nonsusceptible pneumococcal strains, their susceptibilities, detected mutations, and efflux[a]

Strain	City	Hospital	Age (yr)	PFGE type	Sero-type	MIC (μg/ml)[b]							Amino acid change(s)			
						Peni	Amox-Clav	Ery	Cipro	Levo	Gemi	Gati	Moxi	GyrA	GyrB	ParC	ParE
2527	Zagreb	University Hospital for Lung Diseases	63	A	23F	4.0	2.0	0.016	16	16	0.125	4.0	2.0	S81Y		S79F	I460V
2529	Zagreb	University Hospital for Lung Diseases	20	A	23F	4.0	2.0	0.016	16	16	0.25	4.0	4.0	S81Y		S79F	I460V
2536	Zagreb	University Hospital for Lung Diseases	41	A	23F	2.0	2.0	0.016	16	16	0.125	4.0	2.0	S81Y		S79F	I460V
2538	Zagreb	University Hospital for Lung Diseases	68	A	23F	2.0	2.0	0.016	8.0	16	0.125	4.0	2.0	S81Y		S79F	I460V
2542	Zagreb	University Hospital for Lung Diseases	64	A	23F	4.0	2.0	0.016	16	16	0.25	4.0	2.0	S81Y		S79F	I460V
4026	Varazdin	Public Health Institute	44	A	23F	2.0	2.0	0.016	**>32**	32	0.5	8.0	2.0	S81Y		S79F	I460V
2578	Cakovec	Public Health Institute	75		23F	2.0	1.0	≤0.008	8.0	16	0.125	4.0	4.0	S81Y		S79F	I460V
4151	Zagreb	Clinical Hospital Center Zagreb	53		14	2.0	2.0	0.03	**8.0**	2.0	0.125	0.5	0.25			S79F	I460V
4205	Zagreb	University Hospital of Infectious Diseases	35	B2	NT	0.03	0.03	0.03	8.0	4.0	0.06	0.5	0.25			D83N	
4214	Split	Public Health Institute	59	B	NT	0.03	0.03	0.03	4.0	**8.0**	0.125	0.5	0.25				I460V
4222	Split	Public Health Institute	33		11	0.03	0.03	0.03	4.0	**8.0**	0.06	0.5	0.25				I460V
4227	Zagreb	University Hospital of Infectious Diseases	21	B	9	2.0	4.0	0.03	4.0	**4.0**	0.06	0.5	0.25		K137N		I460V
4229	Zagreb	Sveti Duh General Hospital	17		15	0.5	0.25	>64	**4.0**	4.0	0.06	0.5	0.25				I460V
4329	Split	University Hospital Split	62		NT	0.016	0.016	0.016	4.0	4.0	0.125	0.5	0.5		K137N		
4838	Zagreb	Public Health Laboratory	73	B1	11	0.03	0.03	0.03	8.0	4.0	0.06	1.0	0.5				I460V
4912	Zagreb	University Hospital Sestre Milosrdnice	58	B1	11	0.03	0.06	0.03	4.0	4.0	0.06	1.0	0.5				I460V
4941	Split	University Hospital Split	18	B3	9	4.0	2.0	0.06	4.0	4.0	0.06	0.5	0.25			S79F, K137N	I460V
4943	Zagreb	University Hospital Sestre Milosrdnice	53		11	0.016	0.016	0.06	**4.0**	4.0	0.06	0.5	0.25			N91D	
4945	Split	University Hospital Split	67	B1	11	0.03	0.06	0.03	**8.0**	4.0	0.06	1.0	0.5				I460V
4967	Cakovec	Public Health Institute	71	B	14	2.0	2.0	4.0	**4.0**	4.0	0.03	0.5	0.25				I460V
4984	Cakovec	Public Health Institute	58		9	≤0.008	0.016	0.03	**4.0**	4.0	0.06	0.5	0.25				

[a] Quinolone nonsusceptibility is defined by a ciprofloxacin MIC of ≥4 μg/ml. Abbreviations: PFGE, pulsed-field gel electrophoresis; Peni, penicillin; Amox-Clav, amoxicillin-clavulanic acid; Ery, erythromycin; Cipro, ciprofloxacin; Levo, levofloxacin; Gemi, gemifloxacin; Gati, gatifloxacin; Maxi, moxifloxacin; NT, nontypeable.
[b] Boldface values indicate the presence of an efflux mechanism.

of our study document an overall 3.6% incidence of ciprofloxacin-nonsusceptible pneumococci in the areas of Croatia studied. Similar to the Canadian experience (20), many strains, belonging to one clone, clustered in a hospital for chronic lung diseases in Zagreb, treating patients for infections such as acute exacerbations of chronic bronchitis. However, several other serotypes and clones were found, both inside and outside Zagreb. In a recent paper from Hungary (7), the rate of highly levofloxacin resistant pneumococcal strains (4.1%) among penicillin-nonsusceptible strains isolated from sputum in a pulmonary department in Budapest was similar to what we found in Croatia among penicillin-susceptible, -intermediate, and -resistant strains. Resistance mechanisms of these strains were not determined.

Analysis of our data showed the presence of two groups of quinolone-nonsusceptible strains. The main difference among these groups was their level of susceptibility to the newer quinolones gatifloxacin, moxifloxacin, and gemifloxacin. The first group was homogeneous in genotype, phenotype, and serotype; however, strains in the second group were heterogeneous, with different types of mutations, serotypes, and pulsed-field gel electrophoresis type. Finally, gemifloxacin had the lowest MICs of all quinolones tested against both quinolone-susceptible and quinolone-nonsusceptible pneumococcal strains.

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