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Emergence and Epidemiology of Fluoroquinolone-Resistant *S. pneumoniae* Isolates From Italy: Report from the SENTRY Antimicrobial Surveillance Program (2001-2004)

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SENTRY ANTIMICROBIAL SURVEILLANCE

ABSTRACT

Background: Fluoroquinolones (FQ) are key antimicrobials for pneumococcal infections. Increased use of FQ should be accompanied by surveillance efforts to monitor the evolution of resistance (R) and patterns of clonal dissemination.
Methods: *S. pneumoniae* (SPN; n=551) collected from 3 Italian centers (Genoa, Catania, Rome) in 2001 - 2004 were susceptibility (S) tested using CLSI broth microdilution methods. Levofloxacin (LEV)- R (>4 µg/ml) isolates were analyzed by ribotyping (RIBO), PFGE, serotyping (SERO) and antimicrobial R pattern (RP). QRDR mutations were characterized by PCR and sequencing.
Results: 83 (15.1%) isolates showed elevated ciprofloxacin MICs (>4 µg/ml; silent *parC* mutations) and 31 (5.6%) were LEV-R. In 2001, all SPN were LEV-S, but R emerged in all sites in 2002. Overall rates of LEV-R in 2002-04 were: Genoa 3.3%, Catania 10.9% and Rome 6.5%. All LEV-R strains showed at least one mutation in *parC*, *parE* or *gyrA*. Each isolate from Genoa had a unique RP, RIBO/PFGE, SERO and QRDR mutation pattern. Two clusters were documented among Rome isolates. Catania isolates (n=19) showed an identical RIBO/PFGE (333.3/A1); but 3 clusters were identified from RP, SERO and QRDR mutation patterns. 5 epidemic clusters are listed in table:

Epidemic cluster	City	RP	RIBO/PFGE	SERO	QRDR mutations		
					parC	*parE*	*gyrA*
A	Catania	PEN^S,MAC$^{I/R}$,TET^S	333.3/A1	9	S79F,D91N,K137N	I460V	S81F
B	Catania	PEN^S,MAC^R,TET^R	333.3/A1	9V	S79F,K137N	I460V	S81F
C	Catania	PEN^S,MAC^R,TET^S	333.3/A1	9	S79F,D91N,K137N	-	S81F
D	Rome	PEN^S,MAC^R,TET^S	255.5/C	19F	S79Y,D91N	I460V	S81F
E	Rome	PEN^S,MAC^R,TET^R	333.3/A1	9	S79F,D91N,K137N	I460V	S81F

One Genoa isolate shared RIBO/PFGE (333.3/A1) and SERO (9) results with Catania and Rome clusters, indicating clonal dissemination.
Conclusions: FQ-R rates increased among SPN in Italian medical centers. Although FQ-R emerged in many epidemiologically distinct strains, clonal dissemination has become a key factor for increasing FQ-R rates.

INTRODUCTION

Streptococcus pneumoniae is a common cause of otitis media, sinusitis, bronchitis, community-acquired pneumonia, and meningitis. Pneumococcal infections occur in very high numbers in the United States (>50 million/year reported in 1996) and elsewhere in the world, especially among elderly patients and children under the age of five, leading to significant morbidity. Administration of a 23-valent pneumococcal vaccine in the adults and a 7-valent vaccine in young children has reduced incidence of invasive pneumococcal disease, while colonization continues to occur. A shift in the serotype distribution toward non-vaccine serotype clusters has been recently reported in the United States and Europe accompanied by a modest reduction in antimicrobial resistance.

Newer fluoroquinolones are a key antimicrobial class in the treatment of pneumococcal infections, with many recently marketed compounds becoming available for the treatment of community-acquired respiratory tract infections with a corresponding increase in drug use. In contrast to other fluoroquinolones, increased use of levofloxacin along with other patient-, institution-, and geographic region-specific factors have been associated with declining pneumococcal susceptibility. Development of higher-level resistance to fluoroquinolones requires sequential mutations, thus a substantial delay may be expected between introduction of fluoroquinolones for treatment and appearance of clinically significant reduction in susceptibility in the overall pneumococcal population. At the individual patient level, however, prior treatment with a fluoroquinolone may result in treatment failure due to quinolone-resistant *S. pneumoniae*. Elderly patients (>65 years of age) are a group at a greater risk of acquiring infections by fluoroquinolone-resistant *S. pneumoniae*. Thus increased use of these agents needs to be accompanied with greater surveillance efforts to monitor emerging resistance, acquisition of mutations via transformation events and clonal dissemination.

Several recent reports have characterized an increased incidence of pneumococcal infections and escalating resistance to penicillins and macrolides in Italy, while maintaining low isolation frequencies of fluoroquinolone-resistant *S. pneumoniae* (1.3 - 2.4%). A SENTRY Antimicrobial Surveillance Program objective studied the incidence, antimicrobial susceptibility patterns and clonal relationships among levofloxacin-resistant pneumococci from medical centers in northwest (Genoa), central (Rome) and southern (Catania) parts of Italy, all rapidly emerging in a relatively short time frame.

MATERIALS AND METHODS

Bacterial isolates. *S. pneumoniae* isolates (n=551) were collected from three medical centers in Italy (Genoa, Catania and Rome) as part of the SENTRY Program (2001-2004). These isolates were from patients with community-acquired respiratory tract infections. Identification of pneumococci was confirmed using standard biochemical tests including bile solubility and colony morphology.

Antimicrobial susceptibility testing. *S. pneumoniae* were tested against >30 antimicrobials including ß-lactams, macrolide-lincosamide-streptogramin$_B$ agents, fluoroquinolones, tetracycline, rifampin, chloramphenicol and trimethoprim/sulfamethoxazole. All strains were tested using reference broth microdilution methods of the Clinical and Laboratory and Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) employing validated dry-form panels made by Trek Diagnostics (Cleveland, OH). Mueller Hinton broth supplemented with 5% lysed horse blood was used as growth medium for susceptibility testing. Where necessary for high-level quinolone minimum inhibitory concentration (MIC) determination, Etests (AB BIODISK, Solna, Sweden) were applied. *S. pneumoniae* ATCC 49619, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were tested in parallel for quality assurance. Antimicrobial susceptibility interpretations were determined based on CLSI criteria.

Epidemiologic typing. Levofloxacin-resistant *S. pneumoniae* were subjected to ribotyping using the automated Riboprinter Microbial Characterization System (Qualicon, Inc.). These patterns were matched to prior database experience patterns by computer analysis and those with ≥ 93% identity were assigned to the same ribogroup. Isolates with matching ribotypes were analyzed by PFGE banding patterns on ethidium bromide stained gels that were examined visually. Isolates showing <3 bands difference were considered identical/clonally related.

The capsular serotypes were determined at the University of Iowa Hygienic Laboratory (Iowa City, IA, USA) by Quelling reaction using type specific antisera produced by Statens Serum Institute (Copenhagen, Denmark).

PCR amplification of QRDR. Amplification of topoisomerase IV and gyrase A gene segments responsible for fluoroquinolone resistant phenotypes were amplified as described by Pestova et al. The primers used were as follows:
parC-F: 5'TGA CAA GAG CTA CCG TAA GTC G 3', *parC*-R: 5'TCG AAC CAT TGA CCA AGA GG 3', *parE*-F: 5'ACG TAA GGC GCG TGA TGA G 3', *parE*-R: 5'CTA GCG GAC GCA TGT AAC G 3', *gyrA*-F: 5'CGT CGC ATT CTC TAC GGA 3', *gyrA*-R: 5'TCT TGC TCA TAC GTG CCT CGG 3'. PCR products were cleaned using the QIAquick PCR purification kit (QIAGEN GmbH, Germany).

The Quinolone Resistance Determining Region (QRDR) amplicons were sequenced using Sanger-based dideoxy sequencing strategy involving the incorporation of fluorescent dye-labeled terminators into the sequencing reaction products. Sequences obtained were subjected to NCBI BLAST search to determine mutations present in the QRDR region of the fluoroquinolone-resistant isolates. QRDR sequences of *S. pneumoniae* S6, a well characterized fluoroquinolone susceptible isolate, were used as an internal control.

RESULTS

- Ciprofloxacin or levofloxacin non-susceptible isolates (MIC, ≥ 4 µg/ml) were essentially non-existent among the Italian SENTRY Program isolates during the years 1997-2000. These resistant strains have emerged in the past four years.
- Isolates from Genoa showed recent greater susceptibility to erythromycin (47.2% in 2001 to 56.1% in 2004), clindamycin (66.7% in 2001 to 72.0% in 2004) and tetracycline (66.7% in 2001 to 77.1% in 2004) whereas a significant decrease in the susceptibilities to erythromycin and clindamycin was noted at the other two Italian sites (Table 1).
- Penicillin susceptibility was high (83.9%, range 88.0 to 96.1%) among the fluoroquinolone-resistant isolates. Only 12.9% of isolates were categorized as intermediate and one isolate (3.2%) as resistant to penicillin, while ceftriaxone susceptibility ranged from 96.9 to 100.0% among ciprofloxacin non-susceptible strains (Table 2), indicating that fluoroquinolone resistance was not associated with ß-lactam co-resistance in these isolates.
- Capsular typing of representative isolates from a cluster or a resistant phenotype from each of the three medical centers revealed four serotypes (9, 15, 19 and 23), with variations in serotypes 9 (V and non-V) and 23 (F and non-F). A total of 71% levofloxacin-resistant pneumococci belonged to serotype 9, and 10 strains among the 11 pediatric patients showed this serotype 9 being clonally related (105.333.3/A1). Serotyping results also supported the epidemiologic data in the Rome isolates (Table 3), whereas it provided further discrimination of the larger epidemic cluster in Catania (333.3/A1). Serotyping results of Genoa isolates reinforced the ribotyping/PFGE data suggesting the non-clonal origin of these resistant pneumococci.
- Ribotyping and PFGE were complimentary epidemiological tools in the clonality studies of *S. pneumoniae* by serotyping to further discriminate related isolates. All six levofloxacin-resistant *S. pneumoniae* isolates from Genoa belonged to different ribotype/PFGE/serotype groups (Table 4). Many isolates carried a S81F mutation in *gyrA*, and a S79F mutation in *parC*.
- All 19 levofloxacin-resistant *S. pneumoniae* isolates from Catania belonged to the same ribotype and a very similar or identical PFGE pattern. However, based on serotyping and mutation analysis as well as resistance patterns, these isolates could be classified into three epidemic clusters as shown in Table 3. Isolates belonging to serotype 9 non-V (*parC* S79F, D91N, K137N; *gyrA* S81F) were found in clusters A and C. Cluster A consisted of tetracycline-susceptible isolates with the I460V mutation in *parE* whereas cluster C isolates were resistant to tetracycline with no mutation in *parE*. Serotype 9V isolates (cluster B) were penicillin-susceptible, but resistant to erythromycin, clindamycin and tetracycline.
- Two separate clusters (D and E) were detected in Rome, but separated in time. Cluster D appeared in year 2002 only (four isolates, 18.2%) and showed a distinct ribotype and PFGE pattern (255.5/C).
- Figure 1 illustrates trends of isolation of *S. pneumoniae* isolates with reduced susceptibility to ciprofloxacin (MIC ≥ 4 µg/ml) at the three Italian medical centers during 2001-2004. Each of the three centers followed different patterns for isolation of fluoroquinolone non-susceptible isolates.

Table 1. In vitro activity of 11 antimicrobial compounds against *S. pneumoniae* isolates from the Italian SENTRY Program sites (2001 - 2004).

Site/year (no. isolates)	MIC$_{50/90}$ µg/ml (% susceptible)								
	Penicillin	Ceftriaxone	Erythromycin	Clindamycin	Tetracycline	Trim/Sulfa[a]	Levofloxacin	Gatifloxacin	Moxifloxacin
Genoa									
2001 (36)	≤0.03/0.06(91.7)	0.03/0.06(100.0)	2/>32(47.2)	≤0.12/>16(66.7)	≤2/>16(66.7)	≤0.5/4(61.1)	1/2(100.0)	0.25/0.5(100.0)	0.12/0.25(100.0)
2002 (41)	≤0.03/0.25(85.4)	0.03/0.25(97.6)	≤0.25/>32(51.2)	≤0.06/>8(62.2)	≤2/>16(63.4)	≤0.5/4(65.9)	1/1(95.1)	0.25/0.5(95.1)	0.12/0.25(94.4)
2003 (57)	≤0.03/0.25(78.9)	0.03/0.5(96.5)	≤0.25/>32(57.9)	≤0.25/>2(68.4)	≤2/>16(61.4)	≤0.5/4(63.2)	1/1(96.5)	0.25/0.5(96.5)	0.12/0.25(96.5)
2004 (82)	≤0.03/0.06(92.7)	0.016/0.06(98.8)	≤0.25/>32(56.1)	≤0.25/>2(72.0)	≤0.25/>8(77.1)	≤0.5/4(70.7)	1/1(97.6)	0.25/0.5(97.6)	0.12/0.25(97.6)
Catania									
2001 (47)	≤0.03/≤0.03(95.7)	≤0.25/≤0.25(95.7)	≤0.25/16(55.3)	≤0.12/≤0.12(95.7)	≤4/≤4(95.7)	2/4(44.7)	1/1(100.0)	0.25/0.25(100.0)	0.12/0.12(100.0)
2002 (55)	≤0.03/≤0.03(96.4)	≤0.25/≤0.25(100.0)	≤0.25/>32(61.8)	≤0.06/>8(70.9)	≤4/>16(72.7)	≤0.5/4(74.5)	1/1(92.7)	0.25/0.5(92.7)	0.12/0.25(93.9)
2003 (67)	≤0.03/0.12(89.6)	0.03/0.25(98.5)	≤0.25/>32(52.2)	≤0.25/>8(68.7)	≤2/>16(70.1)	2/4(43.9)	1/1(92.5)	0.25/0.5(92.5)	0.12/0.25(92.5)
2004 (53)	≤0.03/0.12(83.0)	0.03/0.5(96.2)	32/>32(35.8)	≤0.25/>8(54.7)	≤2/>8(61.5)	≤0.5/4(56.6)	1/>4(81.1)	0.25/4(81.1)	0.12/2(81.1)
Rome									
2001 (21)	≤0.03/≤0.03(95.2)	0.016/0.03(100.0)	≤0.25/8(85.7)	≤0.12/≤0.12(90.5)	≤2/≤2(90.5)	≤0.5/2(85.7)	1/1(100.0)	0.25/0.5(100.0)	0.12/0.25(100.0)
2002 (22)	≤0.03/0.06(95.5)	0.03/0.12(100.0)	≤0.25/>32(68.2)	≤0.06/>8(72.7)	≤2/>16(68.2)	≤0.5/≤0.5(95.5)	1/>4(81.8)	0.25/4(81.8)	0.12/4(81.8)
2003 (41)	≤0.03/2(80.5)	0.016/0.5(100.0)	≤0.25/>32(63.4)	≤0.25/>2(75.6)	≤2/>16(75.6)	≤0.5/>4(85.4)	2/2(97.6)	0.5/0.5(97.6)	0.12/0.25(97.6)
2004 (29)	≤0.03/≤0.03(93.1)	0.03/0.12(96.6)	8/>32(44.8)	≤0.25/>2(58.6)	ND[a]	≤0.5/4(86.2)	1/1(96.6)	0.25/0.5(96.6)	0.12/0.12(96.6)

a. ND = not determined and trim/sulfa = trimethoprim/sulfamethoxazole.

Table 2. Overall (Italy, 2001-2004) susceptibilities of *S. pneumoniae* isolates with reduced susceptibilities to ciprofloxacin (MIC, ≥ 4 µg/ml).

Site (no. isolates)	Antimicrobial agent (% susceptible)								
	Penicillin	Ceftriaxone	Erythromycin	Clindamycin	Tetracycline	Trim/Sulfa	Levofloxacin	Gatifloxacin	Moxifloxacin
Genoa (25)	88.0	100.0	60.0	72.0	60.0	84.0	76.0	76.0	76.0
Catania (26)	96.1	100.0	19.2	19.2	23.0	92.0	26.9	26.9	22.7
Rome (32)	90.6	96.9	59.4	71.9	71.9	96.9	81.2	81.2	81.2

Table 3. Characteristics of epidemic clusters identified from Catania and Rome (Italy, 2001-2004).

Cluster	Site	Resistance phenotype[a]	Ribotype pattern/PFGE	Serotype	QRDR mutations		
					parC	*parE*	*gyrA*
A	Catania	PEN^S, MAC$^{I/R}$, TET^S	333.3/A1	9 not V	S79F,D91N,K137N	I460V	S81F
B	Catania	PEN^S, MAC^R, TET^R	333.3/A1	9V	S79F,K137N	I460V	S81F
C	Catania	PEN^S, MAC^R, TET^S	333.3/A1	9 not V	S79F,D91N,K137N	-[b]	S81F
D	Rome	PEN^S, MAC^R, TET^S	255.5/C	19F	S79Y,D91N	I460V	S81F
E	Rome	PEN$^{S/R}$, MAC^R, TET^R	333.3/A1	9 not V	S79F,D91N,K137N	I460V	S81F

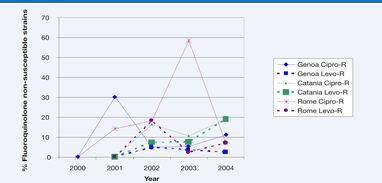
a. PEN = penicillin, MAC = erythromycin/clindamycin, TET = tetracycline, S = susceptible, I = intermediate and R = resistant.
b. No mutations identified.

Table 4. Antimicrobial susceptibility profiles and epidemiologic variability of fluoroquinolone-resistant *S. pneumoniae* isolates from Genoa.

Isolate #	Year	Resistance phenotype	Ribotype	QRDR mutations			MIC (µg/ml)							
				parC	*parE*	*gyrA*	Penicillin	Ceftriaxone	Erythromycin	Clindamycin	Tetracycline	Trim/Sulfa	Gatifloxacin	Moxifloxacin
3749	2002	RP1	258.255.4	S79F	I460V	S81F	≤0.03	0.016	>32	>8	>16	≤0.5	4	2
2395	2004	RP2	258.254.2	S79F,D91N	-[a]	S81F	≤0.03	0.016	≤0.25	≤0.25	0.5	≤0.5	4	2
2447	2003	RP2	258.257.2	S79Y,D91N	-	E85K	≤0.03	0.06	≤0.25	≤0.25	≤2	≤0.5	>4	4
2366	2004	RP3	258.254.1	S79F,D91N,K137N	I460V	S81F	0.25	0.12	≤0.25	≤0.25	0.25	≤0.5	>4	4
3724	2002	RP4	105.339.8	D83G	I460V	S81F	0.06	0.06	≤0.25	≤0.06	≤2	2	4	2
2411	2003	RP6	105.333.3	S79F,D91N,K137N	-	S81F	0.12	0.06	≤0.25	≤0.25	>16	≤0.5	4	2

a. - = No mutations identified.

Figure 1. Occurrence of fluoroquinolone resistant (R) *S. pneumoniae* isolates from three medical centers in Italy. —— ciprofloxacin MIC of ≥ 4 µg/ml (Cipro-R); ----- levofloxacin MIC of ≥ 4 µg/ml (Levo-R).



CONCLUSIONS

- Resistance to fluoroquinolones in Italian medical centers (3) has rapidly emerged with a diverse array of genetic backgrounds, but evidence exists that the selection of fluoroquinolone resistance within successful multi-drug resistant clones led to dissemination of resistance in some communities. In Genoa all the isolates were epidemiologically distinct whereas clonality played a key role at the other medical centers.
- Some of the earlier Italian isolates from Genoa and Rome predominantly had the I460V mutation in *parE*, also present in a large number of levofloxacin-resistant isolates in later years. Mutations in *parE* are regarded as "benign" by some investigators, however recent experience clearly demonstrates that isolates with this mutation alone can be more resistant to norfloxacin and ciprofloxacin than isolates with no amino acid substitution leading to low level fluoroquinolone resistance. Other mutations in *parC* (D435N and S79L) and *gyrA* (S81I or Y) described elsewhere were absent in the Italian isolates tested.
- It remains possible that current dissemination of fluoroquinolone resistance in pneumococci may be a result of the change of this drug class to first line use as treatment of community-acquired respiratory tract infections.
- Higher rates of levofloxacin resistance have been reported among penicillin non-susceptible isolates in USA and Europe. However, only one of the fluoroquinolone-resistant *S. pneumoniae* isolates in our study was resistant to penicillin or ceftriaxone. Parenteral cephalosporins with or without co-drugs are regarded as potent and safe options for use in hospitalized patients with community acquired pneumococcal pneumonia. Thus in the era of increasing fluoroquinolone resistance, it may be prudent to use potent ß-lactams or combination therapeutic approaches to reduce development and dissemination of fluoroquinolone-resistant pneumococci, especially in nations (Italy) or metropolitan areas where ß-lactam resistance remains limited.

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