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Increasing resistance of *Streptococcus pneumoniae* to fluoroquinolones: results of a Hong Kong multicentre study in 2000

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The MICs of 13 antimicrobial agents including seven fluoroquinolones (ciprofloxacin, levofloxacin, sparfloxacin, grepafloxacin, gatifloxacin, moxifloxacin and clinafloxacin) for *Streptococcus pneumoniae* isolates obtained from all regions of Hong Kong in the year 2000 were determined by the Etest. Overall, 39.4% of 180 isolates were susceptible to penicillin, 11.7% were intermediate and 48.9% were resistant. The overall prevalence of fluoroquinolone non-susceptibility (levofloxacin MIC ≥ 4 mg/L) was 13.3% but increased to 27.3% among the penicillin-resistant isolates. For the fluoroquinolone non-susceptible isolates, within-class cross-resistance was common. For the fluoroquinolone non-susceptible isolates, the median MICs of clinafloxacin, gatifloxacin, moxifloxacin, sparfloxacin and grepafloxacin were, respectively, six-, 24-, 32- 84- and 128-fold higher than those for the susceptible isolates. All fluoroquinolone non-susceptible strains were derived from adults. The prevalence of fluoroquinolone resistance was higher in isolates from older patients (17.1% among those ≥ 65 years of age versus 9.1% among those 18–64 years of age, $P < 0.001$) and from adults with chronic obstructive pulmonary disease (24.6% versus 9.3%, $P = 0.01$). All fluoroquinolone non-susceptible strains were non-susceptible to penicillin (MIC range 2–4 mg/L), cefotaxime (MIC range 1–4 mg/L) and erythromycin (MIC range 4–≥ 256 mg/L). The fluoroquinolone non-susceptible isolates were genetically related to the Spain^{23F}-1 clone when analysed by pulse-field gel electrophoresis and multilocus sequence typing. In conclusion, a rapid increase in the prevalence of fluoroquinolone resistance among *S. pneumoniae* was found in Hong Kong. Typing analysis suggests that this is due to the pan-regional dissemination of a fluoroquinolone-resistant variant (designated Hong Kong^{23F}-1) of the globally distributed Spain^{23F}-1 clone.

Introduction

The upsurge of multiply resistant strains of *Streptococcus pneumoniae* in recent years has spawned interest in the use of fluoroquinolones to treat respiratory tract infections. Many agents in this antimicrobial class have been evaluated both experimentally and clinically. The newer members have been termed 'respiratory fluoroquinolones' or 'third generation fluoroquinolones' because of their superior activities against Gram-positive cocci compared with the older fluoroquinolones.[1] In recent surveys involving large numbers of *S. pneumoniae* isolates, the newer agents gatifloxacin and moxifloxacin were generally eight- to 16-fold more potent than older agents such as ciprofloxacin, ofloxacin and levofloxacin. Among the pneumococci, resistance to the respiratory fluoroquinolones remains rare

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in most countries but continued surveillance is required, particularly in regions where there are strains showing reduced susceptibility to the older fluoroquinolones. Hong Kong, together with Ireland, Canada and Spain, are regions that have reported increasing rates of fluoroquinolone resistance among the pneumococci.[2–5] In Hong Kong, for example, the percentage of *S. pneumoniae* resistant to fluoroquinolones has increased from <0.5% in 1995 to 5.5% in 1998.[4] The purpose of the present study was to evaluate the comparative activities of fluoroquinolones including four recent fluoroquinolones (grepafloxacin, gatifloxacin, moxifloxacin and clinafloxacin) against contemporary isolates of *S. pneumoniae* from Hong Kong.

Materials and methods

Bacterial isolates and patient data

During a 4 month period from January to April 2000, isolates of *S. pneumoniae* were collected from six participant hospital laboratories located in widely separated areas of Hong Kong. These hospitals cover over half of the population in this region. Each laboratory submitted 30 consecutive, non-duplicate isolates of *S. pneumoniae* derived from clinical samples. Isolates obtained from the same patient during the same episode of infection were only included once. From each laboratory only one isolate per patient accompanied by relevant strain-specific patient data (date of isolation, site of bacterial isolation, age, gender, patient identifying number and co-morbidity) was referred for study. Co-morbidity was defined as any underlying illness that might increase the risk of infection. All isolates were subcultured and re-identified using colony morphology, Gram's stain, optochin susceptibility and bile solubility. Isolates were stored at −20°C until tested in batches. Only pure culture isolates were included in the final study. During the study period, the infection control service in the hospitals did not identify any clustering of pneumococcal diseases.

Antimicrobial agents

Etest strips of penicillin, amoxicillin (as co-amoxiclav 2:1), erythromycin, cefuroxime, cefotaxime, quinupristin/dalfopristin, ciprofloxacin, levofloxacin, sparfloxacin, grepafloxacin, gatifloxacin, moxifloxacin and clinafloxacin were purchased from AB Biodisk, Solna, Sweden. Moxifloxacin and levofloxacin powders with known potencies were kindly provided by Bayer China Company, Ltd, Hong Kong, China and the R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ, USA, respectively.

Determination of MICs and interpretation

Etest MICs were determined according to the manufacturer's instructions. All susceptibility testing was carried out in a single laboratory at the University of Hong Kong. Test inocula were prepared from pneumococcal colonies grown on sheep blood agar that had been incubated for 20–24 h in 5% CO_2. Colonies were suspended in 0.9% saline to obtain a suspension equivalent to the turbidity of a 0.5 McFarland standard. From this suspension, Etests were performed on Mueller–Hinton agar with 5% sheep blood (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). The plates were incubated at 35°C in 5% CO_2 for 20–24 h. MICs falling between two marks on the Etest strip were rounded up to the next highest two-fold dilution, as recommended in the instructions. Broth microdilution tests were performed using the procedures described by the NCCLS. Cation-adjusted Mueller–Hinton broth (Difco, Detroit, MI, USA) supplemented with 5% horse blood was used as the test medium. Test inocula were prepared as above and further diluted within 15 min to provide a final inoculum density of 5×10^5 cfu/mL in the wells of the microdilution panels. For all MIC determinations, the bacterial inocula were validated by back titration in 10% of the tests to ensure the desired inoculum density. Quality control strains (*S. pneumoniae* ATCC 49619, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922) were included with each run. Interpretation of results was according to published breakpoints of the NCCLS.[6] For ciprofloxacin, the criteria were: susceptible, ≤2 mg/L; resistant, ≥4 mg/L.[2] Fluoroquinolone non-susceptible pneumococci were defined as those for which the MIC of levofloxacin was at least 4 mg/L.

Typing of isolates

The subset of 24 isolates with reduced susceptibility to levofloxacin was examined further by serotyping, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). Serotyping of the isolates was determined by the Quellung reaction[7] using sera with various reactivities from the Statens Seruminstitut (Copenhagen, Denmark). Protocols for PFGE analysis and MLST have been described previously.[8,9] *Sma*I was used for digestion of DNA in the PFGE analysis and the results were interpreted according to a scheme suggested by Tenover *et al.*[10] Strain SP264 (kindly provided by K. P. Klugman), representative of the Spain^{23F}-1 clone was used as a control in the PFGE and MLST analysis.

Statistical analysis

The χ^2, Fisher's exact or the Kruskal–Wallis test was used for statistical analysis. A P value of <0.05 was considered significant.

Results

The number of isolates obtained from different age groups was as follows: <2 years (four); 2–5 years (six); 6–17 years

(nine); 18–49 years (23); 50–64 years (21) and ⩾65 years (117). Of the isolates, 156 (86.7%) were from sputum, eight from tracheal aspirate, three from blood, two from bronchoalveolar lavage fluid and 11 from eye, ear or nose. The patient's co-morbidity was provided for 172 (95.6%) isolates. One or more co-morbidity was present in 59.9% (103/172) of the patients. The most common was chronic obstructive pulmonary disease (COPD) (57), followed by neurological disease (20), malignancy (18), heart disease (10), diabetes mellitus (eight), bronchiectasis (four) and systemic lupus erythematosis (two).

The susceptibilities of the 180 pneumococcal isolates to 13 antimicrobial agents are summarized in Table 1. The MICs of amoxicillin were generally identical or within a two-fold dilution of that of penicillin. High MICs of penicillin (4 mg/L), cefotaxime (4 mg/L) and erythromycin (32–⩾256 mg/L) were found in 11.1% (20/180), 3.9% (7/180) and 25.6% (46/180) of the isolates, respectively. Rates of penicillin non-susceptibility were not significantly different between the various age groups. Penicillin MICs (geomean ± S.D.) were highest in hospital A (0.66 ± 1.1 mg/L), followed by hospitals C (0.51 ± 1.1 mg/L), E (0.5 ± 1.1 mg/L), B (0.23 ± 1.1 mg/L), F (0.21 ± 1.1 mg/L) and D (0.14 ± 0.7 mg/L) (Kruskal–Wallis test, $P = 0.001$). Between 12.2 and 17.8% of the strains were intermediately resistant or resistant to the seven fluoroquinolones. All fluoroquinolone non-susceptible strains were non-susceptible to penicillin (MIC range 2–4 mg/L), cefotaxime (MIC range 1–4 mg/L) and erythromycin (MIC range 4–⩾256 mg/L).

All fluoroquinolone non-susceptible isolates were from adults. Frequency of isolation was dissimilar in the hospitals (nine in A, three in B, four in C, one in D, six in E and one in F, $P = 0.02$). The prevalence of fluoroquinolone non-susceptibility was higher in isolates from older patients [17.1% (20/117) among those ⩾65 years of age compared with 9.1% (4/44) among those 18–64 years of age, $P < 0.001$] and from adults with COPD [24.6% (14/57) compared with 9.3% (9/97), $P = 0.01$]. Fluoroquinolone non-susceptibility was not associated with the other co-morbidities. The MIC distribution of the seven fluoroquinolones for levofloxacin-susceptible and non-susceptible isolates is shown in Table 2. For fluoroquinolone-susceptible isolates, the rank order of potency (MIC_{50}/MIC_{90} in mg/L) was as follows: clinafloxacin (0.094/0.125) > moxifloxacin (0.125/0.19) > gatifloxacin (0.25/0.38) > grepafloxacin (0.25/0.5) > sparfloxacin (0.38/0.5) > levofloxacin (0.75/1) > ciprofloxacin (1/2). For the levofloxacin non-susceptible isolates, within-class cross-resistance was common. For the fluoroquinolone non-susceptible isolates, the median MICs of clinafloxacin, gatifloxacin, moxifloxacin, sparfloxacin and grepafloxacin were six-, 24-, 32-, 84- and 128-fold higher than those for the susceptible isolates. For levofloxacin, MICs determined by Etest were generally two- to four-fold higher than those determined by broth microdilution. For moxifloxacin, Etest MICs were identical or within a two-fold dilution difference of those by broth microdilution. Overall interpretive agreement between the two methods was good for the two fluoroquinolones, with no major discrepancy.

The levofloxacin non-susceptible isolates could be divided into three groups on the basis of their capsular serotypes, 23F ($n = 9$), 19F ($n = 10$) or 14 ($n = 5$). However, only one major cluster was identified by molecular analysis. Using MLST it was shown that a single allelic profile (4-4-2-4-4-1-1) was shared by strain SP264 and all 24 isolates. Similarly, by PFGE, 22 isolates gave patterns that were either indistinguishable from or closely related to that of SP264. The remaining two isolates were possibly related to SP264.

Discussion

A marked increase in the overall prevalence of non-susceptibility to the fluoroquinolones was found compared with our previous study with strains obtained by a similar method in 1998.[4] Over a period of <2 years, the prevalence of levofloxacin non-susceptibility increased from 5.5% to 13.3% among all the strains and from 9.2% to 28.4% among the penicillin-resistant strains. Such strains were found in all the participant laboratories involving 10 hospitals located in widely separated areas in Hong Kong. This indicated that levofloxacin non-susceptible *S. pneumoniae* is now widespread in this locality. Although the trend of decreasing susceptibility to fluoroquinolones is similar to that observed by others, studies in other countries have found only a modest increase in the rates of resistance. Chen *et al*. reported a rise in ciprofloxacin-resistant (MIC ⩾ 4 mg/L) pneumococci from 1.5% to 2.9% in Canada between 1993 and 1998.[2] In Spain, pneumococci with the same degree of fluoroquinolone non-susceptibility have increased from 0.9% in 1991–1992 to 3% in 1997–1998. In the USA, fluoroquinolone non-susceptibility (levofloxacin MIC ⩾ 4 mg/L) among pneumococcal samples was 1.2% in 1999 compared with 0.2% in 1997–1998 and ⩽0.03% in 1997.[11–13]

This investigation, in agreement with our earlier study,[14] showed that fluoroquinolone non-susceptibility in pneumococci is associated with COPD and old age. Of the 24 resistant isolates, 20 (83.3%) were isolated from patients aged ⩾65 years and 14 (58.3%) from patients with COPD. COPD patients are a group that are commonly colonized or infected with *S. pneumoniae* and when an exacerbation occurs the colony counts increase,[15] therefore increasing the likelihood of first-step mutants being present. If such mutants are not killed or eradicated, as might happen when patients are treated with a fluoroquinolone with borderline activity, when an inadequate dose is used or in the case of poor absorption, then a higher level of resistance will emerge. Indeed, a history of suboptimal use of fluoroquinolone was common among our patients with fluoro-

Table 1. MICs of 13 antimicrobial agents for *S. pneumoniae* isolates based on susceptibility to penicillin (n = 180)

Drug	Isolate	n	MIC range[a]	MIC_{50}	MIC_{90}	Mode	S (%)	I (%)	R (%)
Penicillin	all	180	0.016–4	1	3	2	39.4	11.7	48.9
	Pen S	71	0.016–0.064	0.023	0.047	0.023	100.0	0.0	0.0
	Pen I	21	0.125–1	0.75	1	1	0.0	100.0	0.0
	Pen R	88	1.5–4	2	4	2	0.0	0.0	100.0
Amoxicillin[b]	all		0.008–4	1	2	1.5	98.3	1.7	0
	Pen S		0.008–0.064	0.023	0.032	0.023	100.0	0.0	0.0
	Pen I		0.023–1.5	0.5	1.5	0.75	100.0	0.0	0.0
	Pen R		0.75–4	1.5	2	1.5	98.6	3.4	0.0
Cefuroxime	all		0.008–⩾32	2	4	3	43.3	1.1	55.6
	Pen S		0.008–0.23	0.023	0.094	0.023	100.0	0.0	0.0
	Pen I		0.016–3	2	3	2	33.3	9.5	57.1
	Pen R		1.5–⩾32	4	6	3	0.0	0.0	100.0
Cefotaxime	all		0.16–4	0.75	2	1.5	45.0	18.3	36.7
	Pen S		0.016–0.25	0.032	0.064	0.032	100.0	0.0	0.0
	Pen I		0.032–1.5	0.75	1	0.75	38.1	57.1	4.8
	Pen R		0.5–4	1.5	2	1.5	2.3	23.9	73.9
Erythromycin	all		0.094–⩾256	6	⩾256	0.19	31.7	0.6	67.8
	Pen S		0.094–⩾256	0.19	⩾256	0.19	71.8	1.4	26.8
	Pen I		0.19–⩾256	⩾256	⩾25	⩾256	9.5	0.0	90.5
	Pen R		0.125–⩾256	8	⩾256	6	4.5	0.0	95.5
Quinupristin/dalfopristin	all		0.38–1.5	0.5	0.75	0.5	100	0.0	0.0
	Pen S		0.38–1	0.5	0.75	0.5	100	0.0	0.0
	Pen I		0.38–1	0.5	1	0.5	100	0.0	0.0
	Pen R		0.38–1	0.5	0.75	0.5	100	0.0	0.0
Ciprofloxacin	all		0.38–⩾32	1	⩾32	1	82.2	–	17.8
	Pen S		0.5–12	1	2	1	95.8	–	4.2
	Pen I		0.38–2	1	2	1	100	–	0.0
	Pen R		0.38–⩾32	1	⩾32	1	67.0	–	33.0
Levofloxacin	all		0.38–⩾32	1	⩾32	0.75	86.7	0.0	13.3
	Pen S		0.5–2	0.75	1	0.75	100.0	0.0	0.0
	Pen I		0.5–1.5	0.75	1	0.75	100.0	0.0	0.0
	Pen R		0.38–⩾32	1	⩾32	1	72.7	0.0	27.3
Sparfloxacin	all		0.094–⩾32	0.38	⩾32	0.38	85.0	2.2	12.8
	Pen S		0.19–0.75	0.38	0.5	0.38	97.2	2.8	0.0
	Pen I		0.094–0.5	0.25	0.5	0.38	100.0	0.0	0.0
	Pen R		0.19–⩾32	0.38	⩾32	0.38	71.6	2.3	26.1
Grepafloxacin	all		0.094–⩾32	0.38	⩾32	0.25	84.4	1.7	13.9
	Pen S		0.19–1.5	0.38	0.5	0.38	98.6	0.0	1.4
	Pen I		0.094–0.5	0.25	0.5	0.25	100.0	0.0	0.0
	Pen R		0.125–⩾32	0.38	⩾32	0.25	69.3	3.4	27.3
Gatifloxacin	all		0.094–⩾32	0.25	6	0.25	87.2	0.6	12.2
	Pen S		0.17–0.38	0.25	0.38	0.25	100.0	0.0	0.0
	Pen I		0.094–0.38	0.19	0.25	0.19	100.0	0.0	0.0
	Pen R		0.125–⩾32	0.25	8	0.25	73.9	1.1	25.0
Moxifloxacin	all		0.047–6	0.125	2	0.125	87.8	3.3	8.9
	Pen S		0.064–0.19	0.125	0.19	0.125	100.0	0.0	0.0
	Pen I		0.047–0.19	0.094	0.19	0.094	100.0	0.0	0.0
	Pen R		0.064–6	0.125	4	0.125	75.0	6.8	18.2
Clinafloxacin	all		0.047–1	0.094	0.5	0.094	–	–	–
	Pen S		0.047–0.19	0.094	0.125	0.094	–	–	–
	Pen I		0.047–0.125	0.094	0.125	0.094	–	–	–
	Pen R		0.047–1	0.094	0.5	0.094	–	–	–

S, susceptible; I, intermediate; R, resistant.
[a]All MIC values are in mg/L.
[b]Co-amoxiclav Etest strips were used. Values refer to the amoxicillin component.

Table 2. Comparative activities of fluoroquinolone against levofloxacin-susceptible and levofloxacin-resistant *S. pneumoniae* clinical isolates

Isolates/fluoroquinolone	MIC (mg/L)																
	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	⩾32
Levofloxacin susceptible (n = 156)																		
ciprofloxacin							3	10	33	64	28	10	3	2	2		1	
levofloxacin							2	9	79	60	2	4						
sparfloxacin			1		17	49	70	16	3									
grepafloxacin			1	2	21	63	52	13	2	1	1							
gatifloxacin			1	4	54	82	13	2										
moxifloxacin		1	2	44	84	23												
clinafloxacin		7	25	107	15	2												
Levofloxacin non-susceptible (n = 24)																		
ciprofloxacin																		24
levofloxacin																	1	23
sparfloxacin										1		1	3	1	2			16
grepafloxacin												3	2	1	1			17
gatifloxacin									1			1	2	1	9	6	2	2
moxifloxacin					1					1	5	1	4	11	1			
clinafloxacin						1	3	16	3	1								

Vertical bars indicate MIC breakpoint for non-susceptibility. Data shown are the number of isolates with the indicated MIC.

quinolone-resistant pneumococci.[14] Such practice might be related in part to confusion regarding the relative potency, dosage and indications of ofloxacin and levofloxacin. For both ofloxacin and levofloxacin, the 100 mg tablet is the only oral formulation available in Hong Kong, and in *MIMS Hong Kong*[16] (an index of essential prescribing information similar to the *British National Formulary* in the UK), the recommended doses of both ofloxacin and levofloxacin for various infections, including respiratory tract infections, were 200–600 mg/day in one to three divided doses. Finally, a drug efflux mechanism is known to be common in local strains of *S. pneumoniae*,[17] which might also facilitate mutational resistance by permitting short-term bacterial survival. In the study by Chodosh *et al.*,[18] the pneumococcal isolates that persisted in two chronic bronchitis patients treated with ciprofloxacin were found to change from susceptible to resistant. Weiss *et al.*[19] also reported a similar finding recently.

In agreement with reports by other investigators,[2,3,5] this and our previous study[14] showed that fluoroquinolone non-susceptibility is associated with resistance to penicillin, cephalosporins, co-trimoxazole and macrolides. Molecular analyses confirmed that this finding is due to the dissemination of a multiply resistant clone that shares an identical MLST allelic profile (4-4-2-4-4-1-1) with the globally distributed strain Spain^{23F}-1. This fluoroquinolone-resistant variant, designated Hong Kong^{23F}-1, was found to have serotype 14 and 19F variants. In contrast, fluoroquinolone non-susceptible strains in Spain and Canada were not clonally related.[2,5]

Dissemination of resistant clones of *S. pneumoniae* has often been associated with increasing antimicrobial usage.[20] In Hong Kong, the most frequently prescribed fluoroquinolones are ofloxacin, ciprofloxacin and levofloxacin (these three fluoroquinolones together constitute >90% of the total usage of this antibiotic class locally). Ofloxacin was licensed for use in this area in 1985, followed by ciprofloxacin in 1988 and levofloxacin in 1994. Data obtained on the inpatient and outpatient use of these three agents from 1994 to 2000 in the Hospital Authority (HA) are shown in the Figure. The HA manages all the public hospitals and specialist outpatient services in this area. Serving a population of 7 million, there are approximately 1 million inpatient discharges, 8 million specialist outpatient attendances and 2 million emergency room attendances each year. Between 1994 and 2000, fluoroquinolone prescriptions increased from 66.8 to 91.8 daily defined doses (DDDs)/1000 persons/year. A breakdown of the usage in terms of clinical indication was not available but we believe that most of the increase is related to increased use for empirical therapy of respiratory tract infections.

Finally, almost all levofloxacin non-susceptible isolates in this study were intermediate or resistant to gatifloxacin and moxifloxacin, making these agents unlikely to be useful in the management of infection by these strains. For isolates with a penicillin MIC of ⩽2 mg/L, appropriate dosing

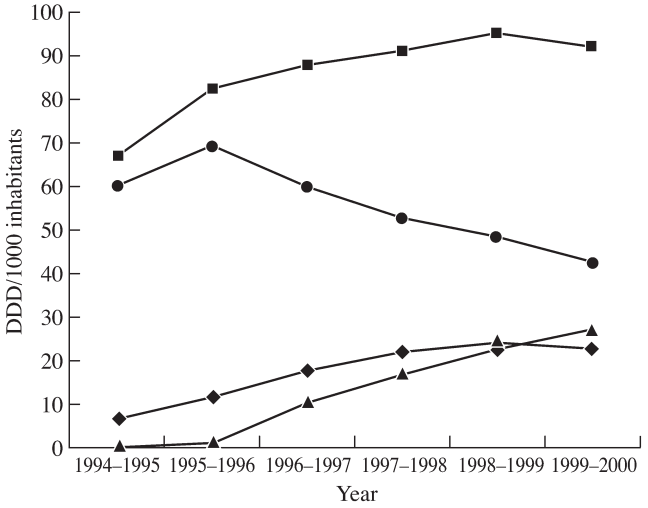

Figure. Usage of fluoroquinolones in Hong Kong. Data shown refer to prescriptions of ciprofloxacin, levofloxacin and ofloxacin in the HA from 1 April 1994 to 31 March 2000. One DDD is equivalent to 0.5 g iv ciprofloxacin, 1 g po ciprofloxacin, 0.4 g ofloxacin (both iv and po), 0.5 g levofloxacin (both iv and po). ◆, Ciprofloxacin; ●, ofloxacin; ▲, levofloxacin; ■, all.

of a suitable β-lactam remains a valid therapeutic option. Owing to a recent revision of the susceptible breakpoint for amoxicillin from 0.5 to 2 mg/L[21] whilst penicillin breakpoints remain unchanged, most (97.2%) of the penicillin non-susceptible *S. pneumoniae* in the present study were categorized as amoxicillin susceptible. For penicillin, the respiratory breakpoint for susceptibility has been suggested to be 2–4 mg/L.[22] In the present sample, 88.9% and 100% of the isolates were inhibited at 2 and 4 mg/L, respectively.

In conclusion, fluoroquinolone resistance in pneumococci has reached disturbingly high rates in Hong Kong both quantitatively and qualitatively. Our data showed that this is related to the emergence and widespread dissemination of a fluoroquinolone-resistant, and multiply-resistant clone. Typing studies suggests that this clone (Hong Kong^{23F}-1) is genetically related to the globally distributed strain Spain^{23F}-1. These findings are of major significance for local patient care, public health and the pharmaceutical industry.

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