Molecular surveillance of rising fluoroquinolone resistance in non-invasive *Streptococcus pneumoniae* isolates in Belgium (1995-2014).

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BACKGROUND

- In the late 90s, respiratory fluoroquinolones were introduced in the treatment of (a)typical pneumonia. However, there was concern that short-term treatment success might be followed by emerging **fluoroquinolone resistant** (FQ-R) *Streptococcus pneumoniae* strains by accumulation of chromosomal mutations.
- In recent years, the role of **efflux** in low-level FQ-R has become more and more appreciated. Apart from a moderate increase in MIC, increased efflux is associated with rising mutational frequencies in the QRDRs [1]. Most clinical relevance is attributed to the reserpine-sensitive heterogenic ABC efflux pump PatAB [2].

Although most *S. pneumoniae* surveillance studies focus on bacteremia, recent work estimated that for every adult bacteremia case there are three non-invasive infections [3]. Here, we present data on FQ-R in **5,602 non-invasive pneumococci,** collected during winter seasons between 1995 and 2014 across 15 Belgian clinical laboratories.

MAIN OBSERVATIONS

(i) Rising resistance to older fluoroquinolones since 2011

All isolates were assessed on FQ-R using broth microdilution (Table 1)

- High-level CIP resistance significantly increased to 9.0% in 2013 (P = 0.00025, X^2 trend analysis incl. Bonferroni's correction).
- Levofloxacin MIC_{50} increased significantly from 0.5 to 1 µg/mL since 2012 (P < 10-6).
- Moxifloxacin is the compound with the highest intrinsic activity; Resistance arises only sporadically, and remained <1% throughout the entire study period.

Table 1 — Yearly percentage of isolates displaying indicated MIC (µg/mL) against three fluoroquinolones. The MIC50 values are coloured orange.

CIPROFLOXACIN		Sensitive (%)			Intermediate resistant (%)				Resistant (%)				
Year	# strains	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	% res.
1995	143	-	0.7	2.8	18.9	34.3	35.7	7.7	-	-	-	-	0.0
1997	162	-	-	-	6.8	17.9	61.7	11.7	2.5	0.6	-	-	3.1
1999	227	-	0.4	0.4	6.2	30.8	47.1	13.2	1.8	-	-	-	1.8
2001	334	-	-	0.9	12.9	38	38	6.6	3	0.6	-	-	3.6
2003	391	-	0.5	3.1	11.3	25.1	46.3	9.5	2.6	1.8	-	-	4.4
2004	424	0.2	1.2	1.9	14.2	37.3	36.3	6.6	2.1	-	0.2	-	2.3
2005	447	0.2	1.1	2.5	12.8	35.6	40.5	6	0.9	0.2	0.2	-	1.3
2006	430	-	0.2	1.4	7.4	28.6	53.7	8.1	0.5	-	-	-	0.5
2007	413	-	0.2	1.5	7.7	30	56.7	1.7	1.5	0.2	0.5	-	2.2
2008	448	-	0.2	0.4	4.7	16.1	73.4	4.7	-	-	0.4	-	0.4
2009	413	-	-	1.9	6.5	44.1	44.1	1.9	1	0.2	0.2	-	1.4
2010	370	-	0.8	2.7	10.8	26.2	55.1	1.9	2.2	-	-	0.3	2.5
2011	368	-	0.3	0.5	4.6	14.9	46.2	29.6	2.2	1.1	0.5	-	3.8
2012	351	-	-	0.3	1.1	14.2	46.4	29.9	7.1	0.6	0.3	-	8.0
2013	369	-	-	-	3	12.5	38.8	36.9	7.3	1.1	0.3	0.3	9.0
2014	312	-	-	-	0.6	9.9	49.7	33	6.4	-	0.3	-	6.7

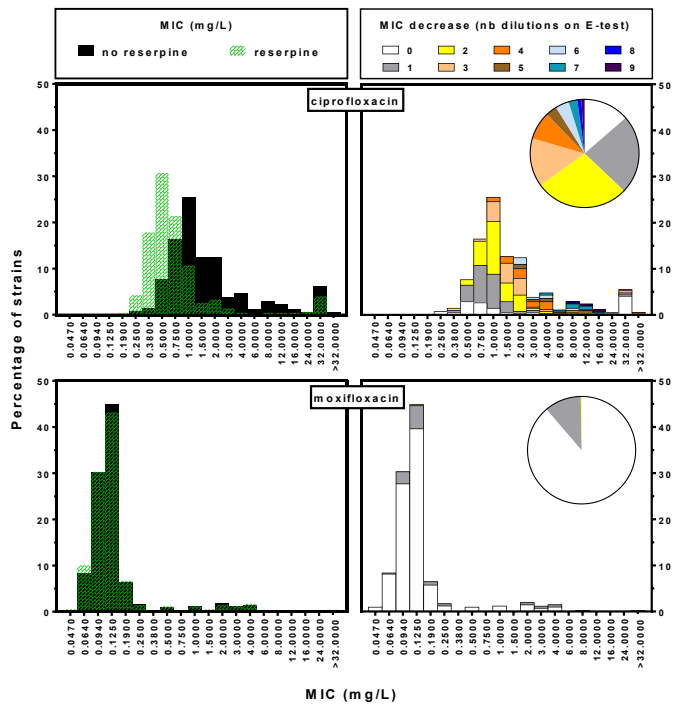
LEVOFLOXACIN		Sensitive (%)							Resistant (%)			
Year	# strains	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	% res.
1995	143	-	1.4	2.8	19.6	40.6	30.1	5.6	-	-	-	0.0
1997	162	-	-	0.6	8	58.6	26.5	4.9	1.2	-	-	1.2
1999	227	-	0.4	-	2.6	37.9	44.1	13.2	1.8	-	-	1.8
2001	334	-	-	1.2	9	47.6	33.2	6.3	2.4	0.3	-	2.7
2003	391	-	0.5	3.6	13.6	31.7	41.4	5.9	1.8	1.5	-	3.3
2004	424	0.5	0.7	3.8	14.2	42.7	30.2	5.2	2.6	-	0.2	2.7
2005	447	0.9	2	4.5	22.6	48.1	15.9	5.4	0.4	-	0.2	0.6
2006	430	0.2	1.2	2.1	9.3	28.6	53.7	8.1	0.5	-	-	0.5
2007	413	0.2	0.5	2.2	13.8	58.1	23.5	0.7	0.2	0.7	-	0.9
2008	448	0.2	-	1.1	6.9	60.7	26.1	4.2	0.2	-	0.4	0.6
2009	413	-	1.2	5.3	30.8	46.2	15	0.7	0.2	0.5	-	0.7
2010	370	0.3	3.5	4.3	17	55.9	15.7	2.4	0.5	0.3	-	0.8
2011	368	0.3	0.5	3	10.1	37	41.3	6.8	0.5	0.5	-	1.0
2012	351	-	-	0.9	3.7	41.3	39	12	2.8	0.3	-	3.1
2013	369	-	-	1.4	2.7	35	49.3	10.3	0.8	0.3	0.3	1.4
2014	312	-	-	0.6	2.2	30.8	59.6	6.1	0.3	-	0.3	0.6

MOXIFLOXACIN		Sensitive (%)							Resistant (%)					
Year	# strains	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	% res.
1995	143	-	9.1	33.6	38.5	13.3	0.7	-	-	-	-	-	-	0.0
1997	162	-	0.6	12.3	38.9	44.4	3.1	0.6	-	-	-	-	-	0.0
1999	227	0.4	1.8	11	40.1	30.4	12.8	2.6	0.9	-	-	-	-	0.9
2001	334	0.6	6.3	9.3	43.7	32.3	5.4	1.5	0.6	0.3	-	-	-	0.9
2003	391	1	6.6	13.6	30.2	36.8	10.5	0.8	-	0.3	0.3	-	-	0.6
2004	424	0.5	4.5	17	39.4	30.2	8	0.2	-	0.2	-	-	-	0.2
2005	447	1.1	4	18.6	39.6	28.2	6.9	1.3	-	0.2	-	-	-	0.2
2006	430	1.8	4.7	17	41.4	30.9	-	0.2	-	-	-	-	-	0.0
2007	413	0.7	2.9	11.1	43.1	30	11.4	-	0.5	0.2	-	-	-	0.7
2008	448	0.2	0.9	7.4	38.6	46.4	6.9	-	-	0.2	-	0.2	-	0.4
2009	413	0.2	5.3	11.1	51.3	25.2	6.3	0.2	0.2	-	-	-	-	0.2
2010	370	-	5.4	11.6	49.5	26.8	5.7	0.8	-	-	0.3	-	-	0.3
2011	368	0.3	3.3	12.8	48.9	27.2	6.5	0.5	0.5	-	-	-	-	0.5
2012	351	-	2.3	5.4	48.1	36.5	6.6	0.9	0.3	-	-	-	-	0.3
2013	369	-	1.4	9.5	53.9	30.1	4.3	-	-	0.8	-	-	-	0.8
2014	312	-	0.3	8.3	52.6	34.3	4.2	-	-	0.3	-	-	-	0.3

(ii) Efflux is pivotal in low-level ciprofloxacin resistance

- For 229 out of 422 isolates (54.2%), at least a twofold decrease in MIC_{CIP} was achieved upon addition of 20mg/L of the efflux pump inhibitor reserpine.
- Moxifloxacin is much less sensitive to reserpine.
- Overexpression of the ABC pump PatAB was found in 48% of reserpine-sensitive strains, but could only be linked to disruptive terminator mutations in a fraction of these (data not shown).

Fig 1 — MIC distribution of ciprofloxacin and moxifloxacin, based on E-test of 422 non-invasive *S. pneumoniae* isolates. Left-hand panels: MIC distributions determined in the absence (control; black) or presence (green) of 20 mg/L reserpine. Right-hand panels: reduction of MIC (in blocks of 0.5 log_2 dilutions from 0 to 3 log_2 dilutions) after addition of 20 mg/L reserpine and plotted as a function of the MIC distribution of the isolates in the absence of reserpine.



(iii) Topoisomerase mutations explain a lot in high-level resistance, but not all

- Classical topoisomerase mutations in *gyrA (n*=25*), parC (n*=46*)* and *parE (n*=3*)* were found in varying combinations, arguing against clonal expansion of FQ-R.
- Marginal impact of recombination with co-habiting commensal streptococci on FQ-R (10.4%)
- A rare combination of DNA Gyrase mutations (GyrA_S81L/ GyrB_P454S) suffices for high-level moxifloxacin resistance, which contrasts the current model.
- We encountered a number of isolates displaying high-level ciprofloxacin resistance, but are deprived of any known molecular resistance mechanism.

Table 2 — Overview of the various FQ-R genotypes encountered in 422 clinical pneumococcal strains. Signature residues of the viridans group of streptococci are shaded in grey.

MIC_{CIP+R} (µg/ml)	MIC_{MOX+R} (µg/ml)	No. isolates	GyrA S81	GyrA E85	GyrA S114	GyrB P454	ParC S52	ParC N91	ParC D78	ParC S79	ParC D83	ParE D435
< 1	0.064-	289	-	-	-	-	-	-	-	-	-	-
(*n*=311)	0.19	10	-	-	G	-	-	-	-	-	-	-
		6	-	-	G	-	G	D	-	-	-	-
		2	-	-	-	-	-	D	-	-	-	-
		2	-	-	-	-	G	D	-	-	-	-
		1	-	-	G	-	G	-	-	-	-	-
		1	F	-	-	-	-	-	-	-	-	-
≤ 2	0.125-	47	-	-	-	-	-	-	-	-	-	-
(*n* = 80)	0.25*	12	-	-	-	-	-	-	-	F	-	-
		1	-	-	-	-	-	-	N	-	-	-
		1	F	-	-	-	-	-	-	-	-	-
		1	-	-	-	-	-	D	-	F	-	-
		2	-	-	-	-	-	-	-	-	N/Y	-
		1	-	-	-	-	-	-	-	F	-	K
		1	-	-	G	-	G	D	-	F	-	-
		5	-	-	G	-	-	-	-	-	-	-
		5	-	-	G	-	G	D	-	-	-	-
		4	-	-	G	-	-	D	-	-	-	-
2-4	0.19-1	3	-	-	-	-	-	-	-	-	-	-
(*n* = 12)		4	-	-	-	-	-	-	-	Y/F	-	-
		1	F	-	-	-	-	-	-	-	-	N
		1	-	-	-	-	-	D	-	F	-	-
		1	-	-	-	-	-	-	-	-	G	-
		1	-	-	G	-	-	-	-	-	-	-
		1	-	-	G	-	G	D	-	-	-	-
> 4	1-32	1	-	-	-	-	-	-	-	-	-	-
(*n* = 29)		3	F	-	-	-	-	-	-	-	-	-
		1	F	-	-	-	-	-	-	-	-	N
		12	F	-	-	-	-	-	-	F/Y	-	-
		2	F	-	-	-	-	-	-	-	G/Y	-
		2	-	-	G	-	-	D	-	F	-	-
		1	Y	-	G	-	-	D	-	Y	-	-
		1	G	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	N	-
		2	-	-	-	-	-	-	-	F/Y	-	-
		1	-	K	-	-	-	-	-	Y	-	-
		1	L	-	G	S	-	-	-	-	-	-

TAKE-HOME MESSAGES

- From 2011 onwards, we observe a trend towards **increased resistance to ciprofloxacin** and (although only visible at the MIC_{50} level) **levofloxacin.** Although a similar increase in ciprofloxacin resistance was reported in Canada, this is not confirmed in other European, American or Asian surveillance studies.
- The **preference of first-step mutations in ParC** is reflected by the 4:1 ratio of single QRDR mutations in the Topoisomerase IV subunits compared to the DNA Gyrase.
- While target topoisomerase mutations and efflux pump (over)expression clearly contribute to FQ-R, we add novel isolates to the existing collection of strains **deprived of known molecular mechanisms of fluoroquinolone resistance.** It would of great value unravel their resistance mechanisms through profound, comparative molecular characterization at the genomic, transcriptomic and proteomic level.

REFERENCES

1. Jumbe, N et al. Antimicrob Agents Chemother 2006; 50: 310-317.
2. El Garch F. et al. J Antimicrob Chemother 2010; 65: 2076-2082.
3. Said MA et al. . PLoS ONE 2013; 8: e60273.

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