Phenotypical and genotypical detection of fluoroquinolone resistance

Christian G. Giske
Associate professor / Consultant physician
Clinical microbiology
Karolinska University Hospital
Santander, 7 October 2011

Detection of resistance beyond clinical breakpoints – why?

- Gram-positives: screening with norfloxacin
- Gram-negatives: nalidixic acid for Enterobacteriaceae – identification of QRDR-mutations in *Salmonella* spp.
- Expanded sometimes also to other Enterobacteriaceae, but without supportive clinical evidence
- Novel resistance mechanisms – not recognized by nalidixic acid – back to ciprofloxacin clinical breakpoints
- Ciprofloxacin clinical breakpoint not sensitive enough to detect emerging plasmid mediated quinolone resistance (PMQR)

Chromosomal resistance mechanisms to fluoroquinolones

Bacteria	ParC (topo IV)	Gyrase (topo II)	Efflux	Phenotype
Gram-positive	+	-	-	NOR R CIP/LEV R
Gram-positive	-	-	+	NOR R CIP R
Gram-positive	+	+		NOR/CIP/LEV R MOX R
Gram-negative	-	-	+	NAL R CIP/LEV r
Gram-negative	+/-	+(+)	-	NAL R CIP/LEV R

Genotypical detection of chromosomal FQ-resistance

Gram-positive

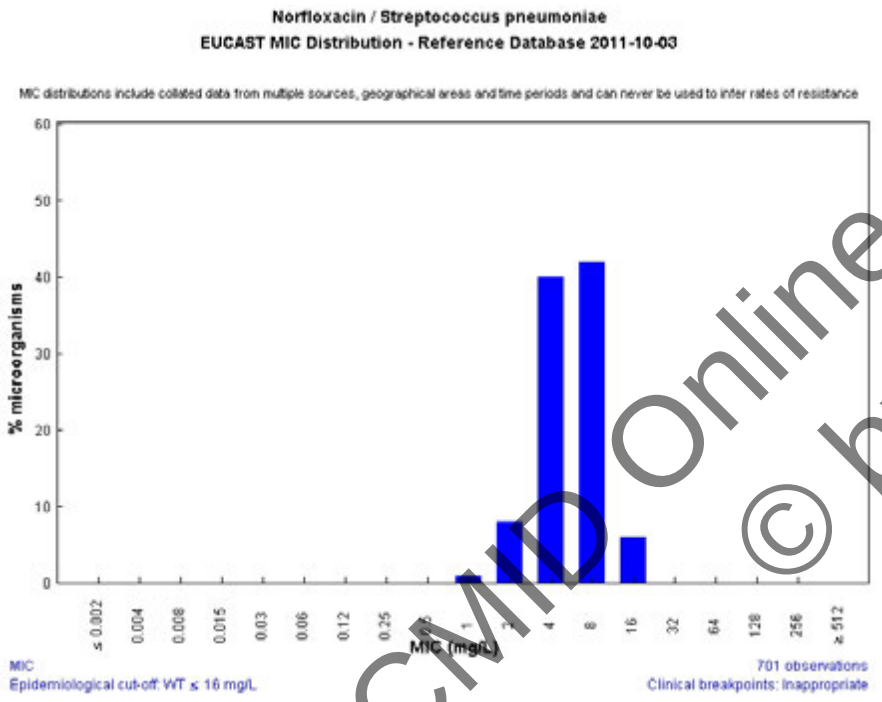
- Detection of mutations in ***parC*** and *gyrA*
- Quantification of mRNA for efflux pumps

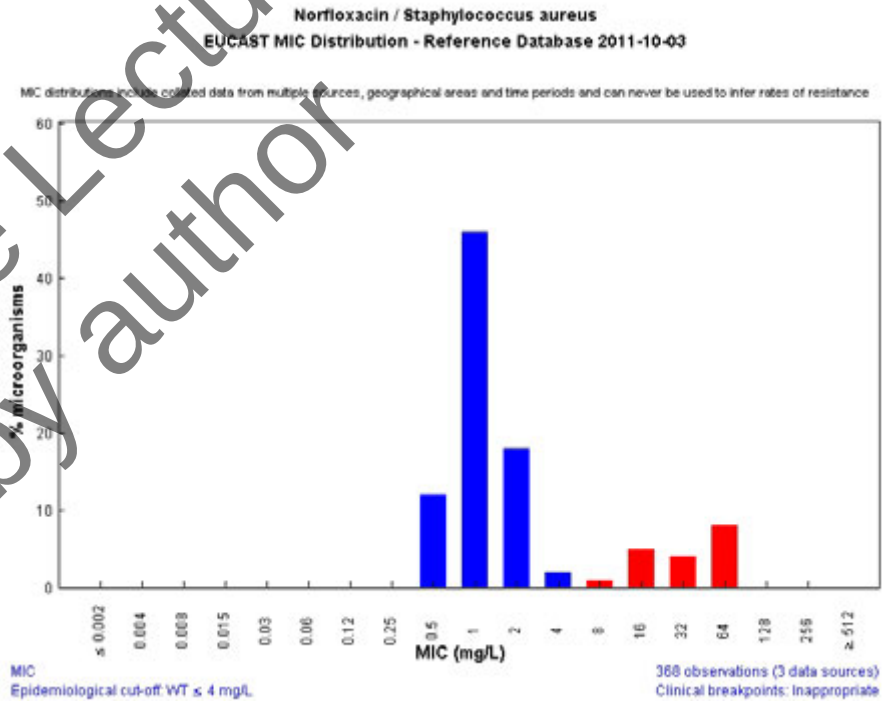
Gram-negative

- Detection of mutations in *parC* and ***gyrA***
- Quantification of mRNA for efflux pumps
- Quantification of mRNA for porins

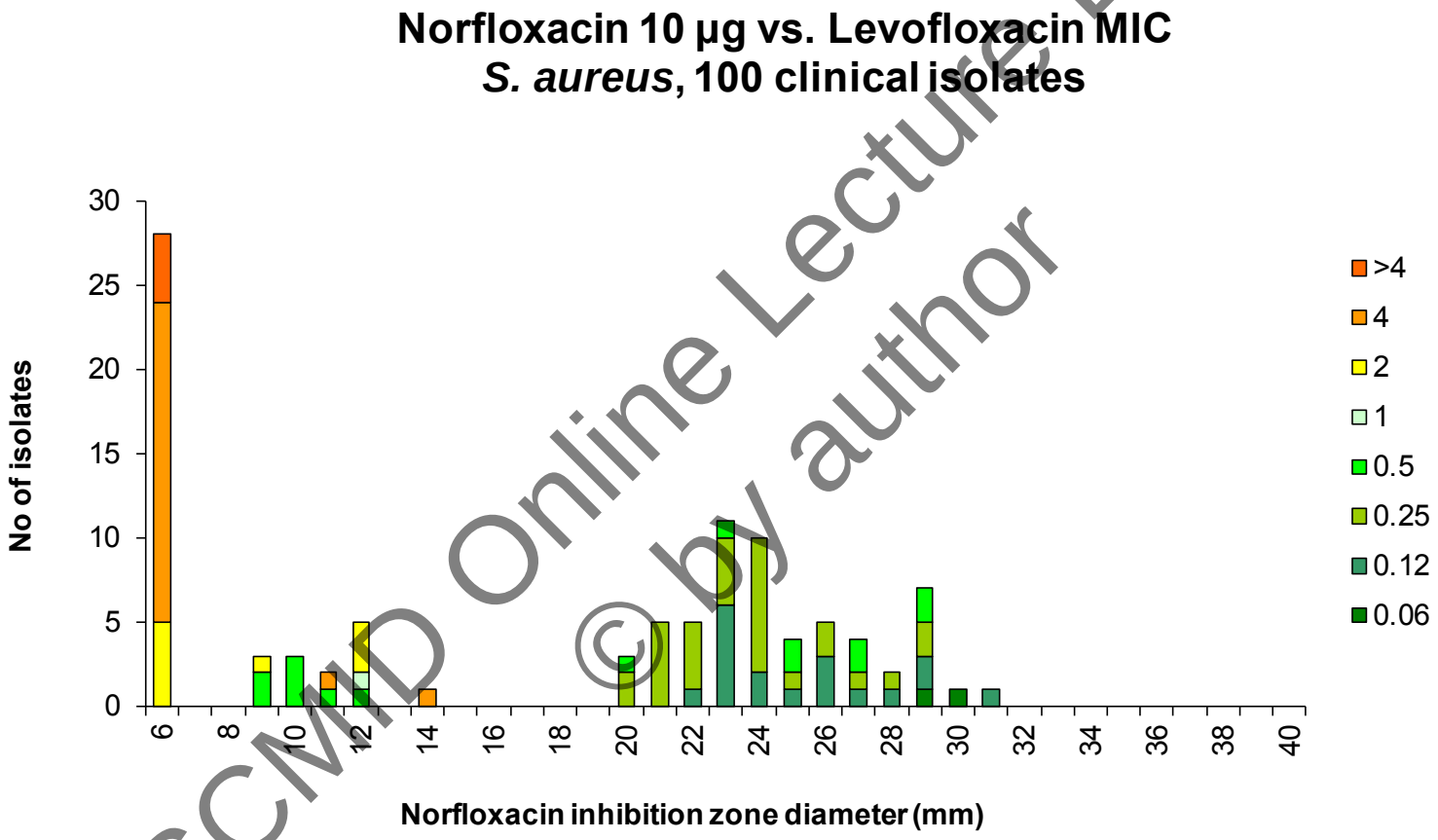
Difficult to confirm susceptibility!

Norfloxacin – a marker of low-grade FQ-resistance in Gram-positive bacteria



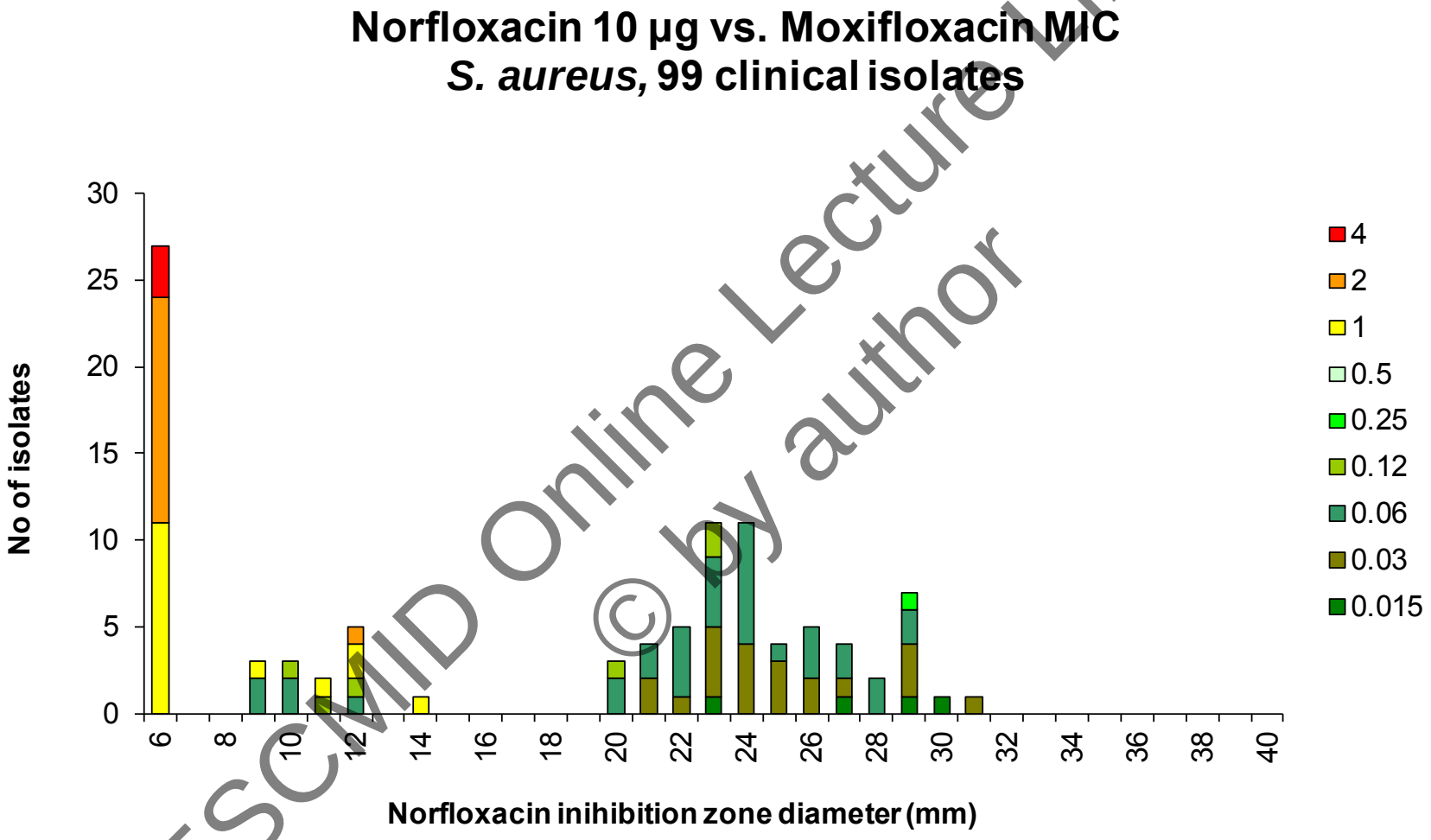


Norfloxacin disk screening



Courtesy of EUCAST, JMI Laboratories (US) and Statens Serum Institut (DK).

Norfloxacin disk screening



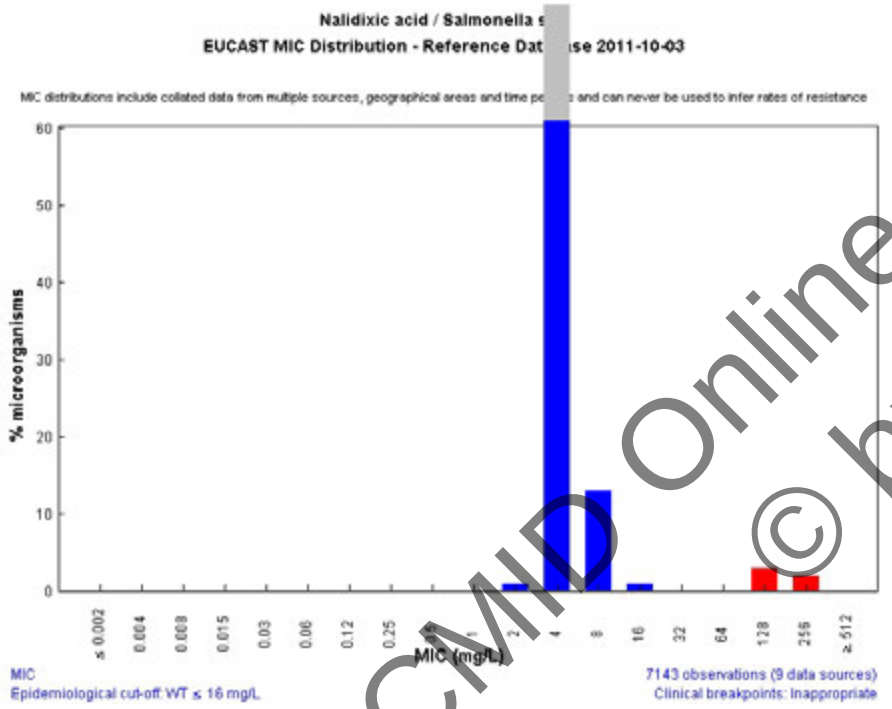
Courtesy of EUCAST, JMI Laboratories (US) and Statens Serum Institut (DK).

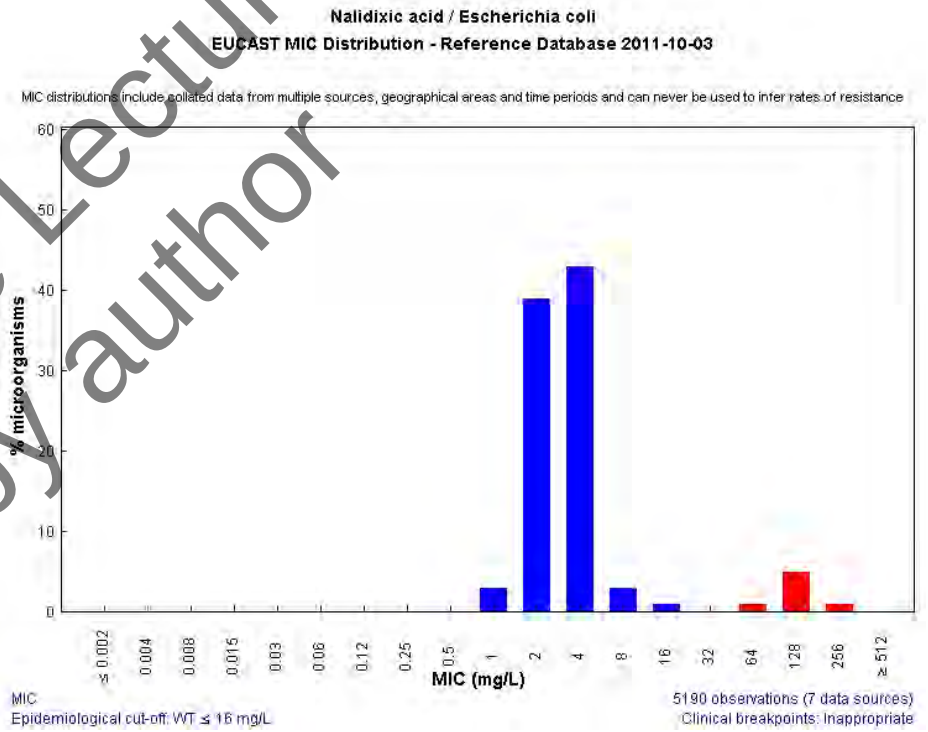
Norfloxacin and *S. pneumoniae*

Strains	Genotype	No. of strains	Mean zone diam[a] (mm) ± SD			
			NOR[b]	CIP	LVX	MXF
WT strains	Wild type	1,151	15 ± 3	24 ± 2	24 ± 2	32 ± 3
LLR mutants	*parC/parE*	46	**6 ± 0**	17 ± 3	20 ± 2	29 ± 3
	parC + efflux	3	**6 ± 0**	**8 ± 3**	17 ± 0	30 ± 1
	Efflux	14	**6 ± 0**	18 ± 2	22 ± 1	29 ± 3
	gyrA	16	15 ± 3	**21 ± 3**	21 ± 2	25 ± 3
HLR mutants	*parC* + *gyrA*	57	6 ± 0	6 ± 1	**7 ± 2**	17 ± 3
	parE + *gyrA*	7	6 ± 0	11 ± 4	**8 ± 2**	20 ± 2

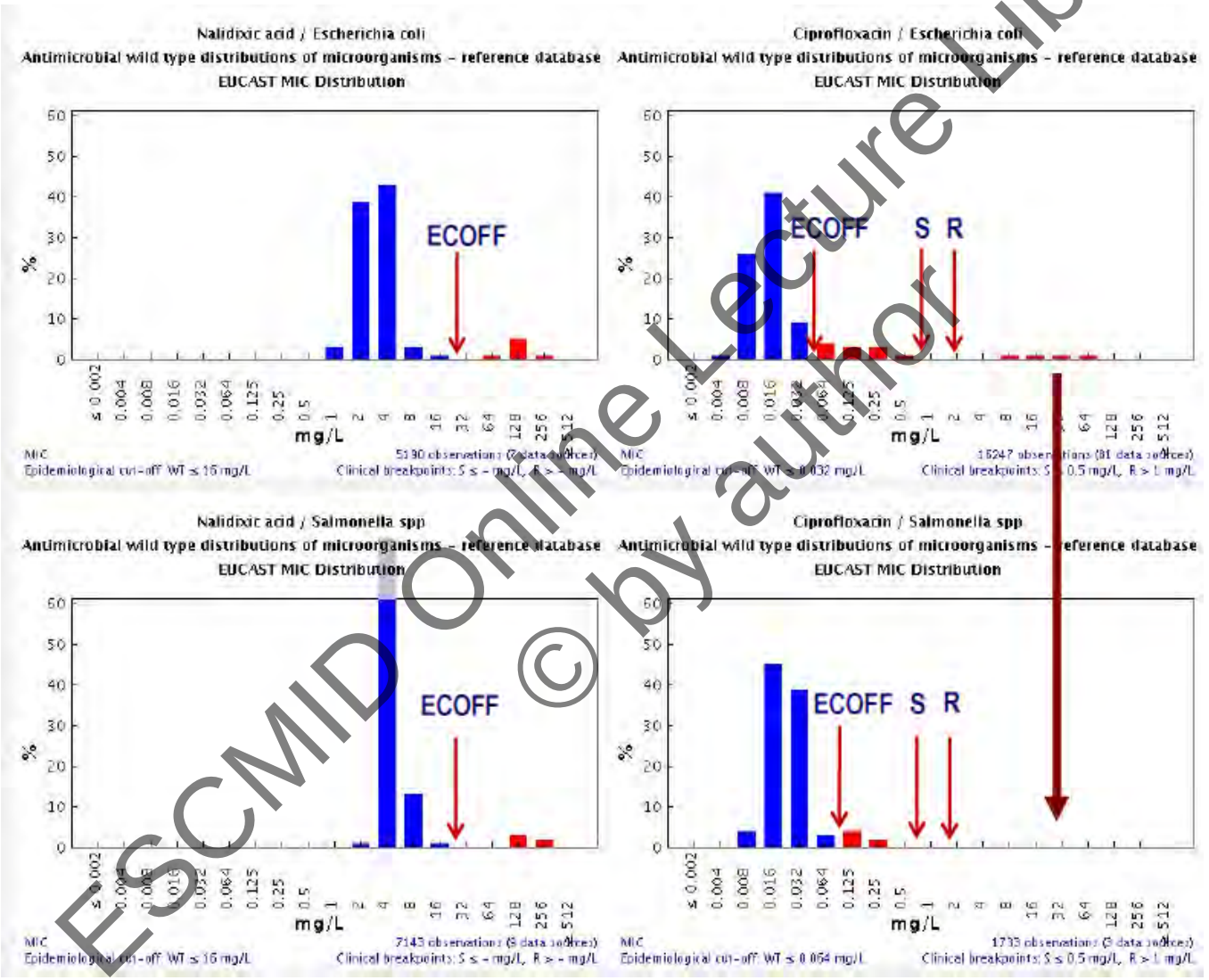
Varon E et al. AAC 2006 Feb;50(2):572-9

Nalidixic acid in Gram-negative bacteria





Epidemiological cut-off values

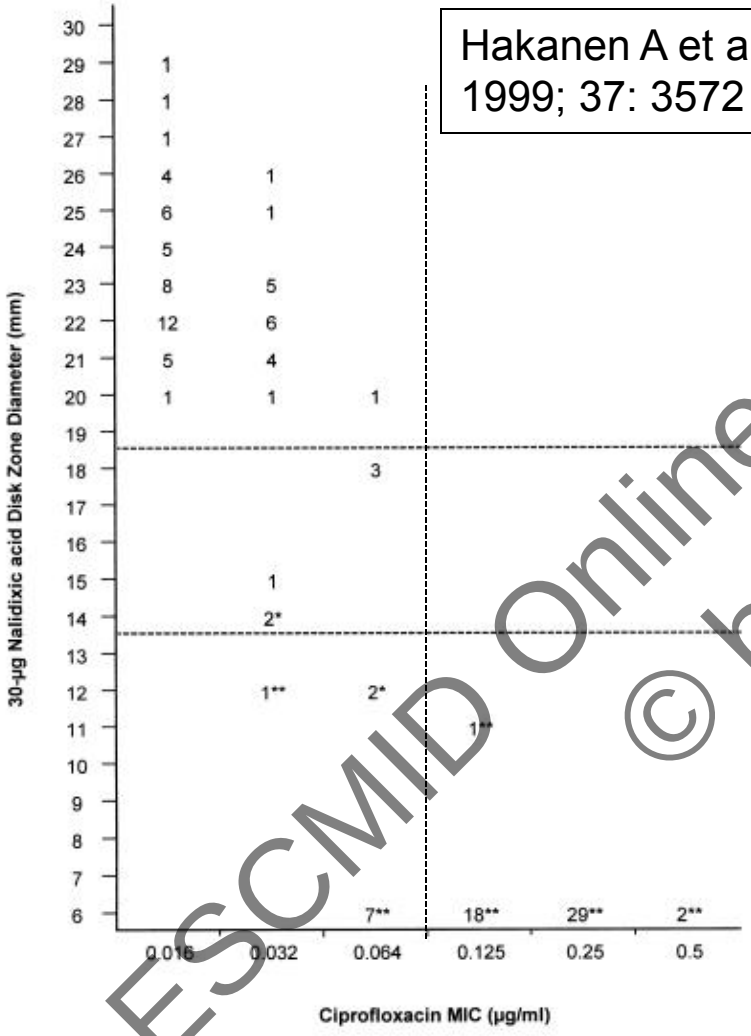


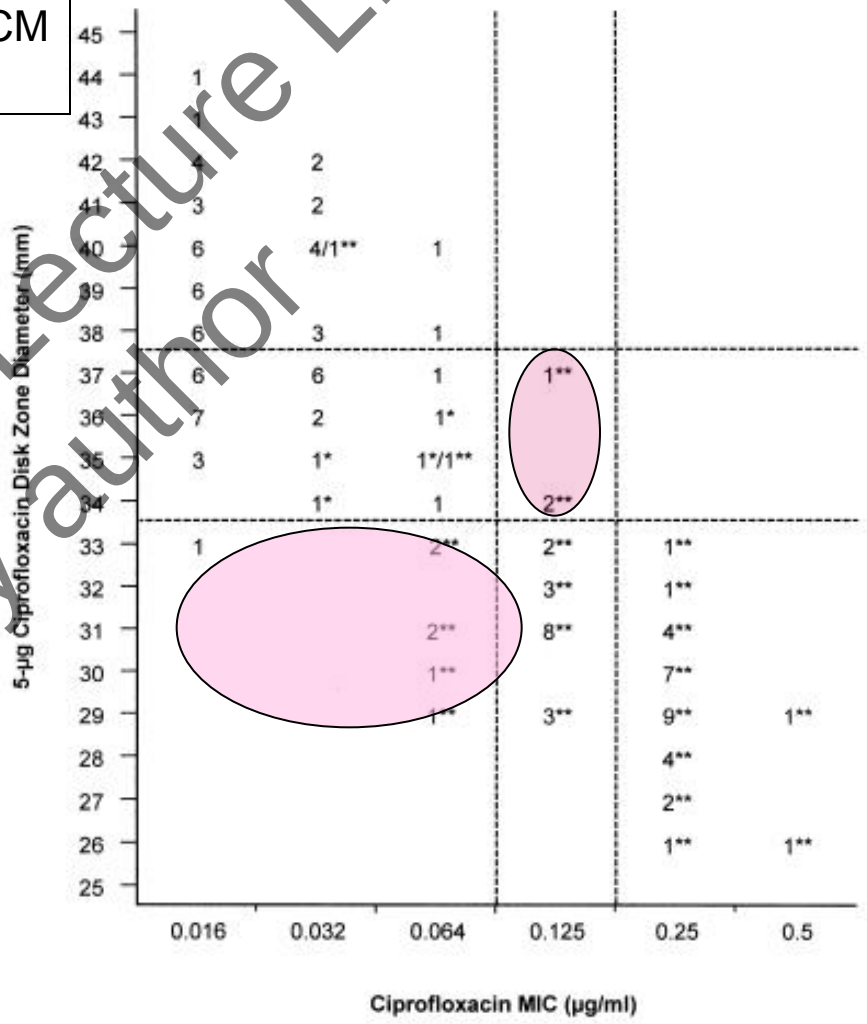
Nalidixic acid and chromosomal resistance

Mutations in			MIC (mg/L)		
gyrA	***parC***	**Efflux**	**NAL**	**LEV**	**CIP**
-	-	-	2	0.06	0.01
+	-	-	**32-256**	0.5	0.5
-	+	-	**64**	0.06	0.01
++	-	-	**>256**	**2**	**1**
+	-	+	**32-256**	**4**	**2**
++	+	-	**>256**	**32**	**64**

Nalidixic acid 30 µg vs ciprofloxacin 5 µg

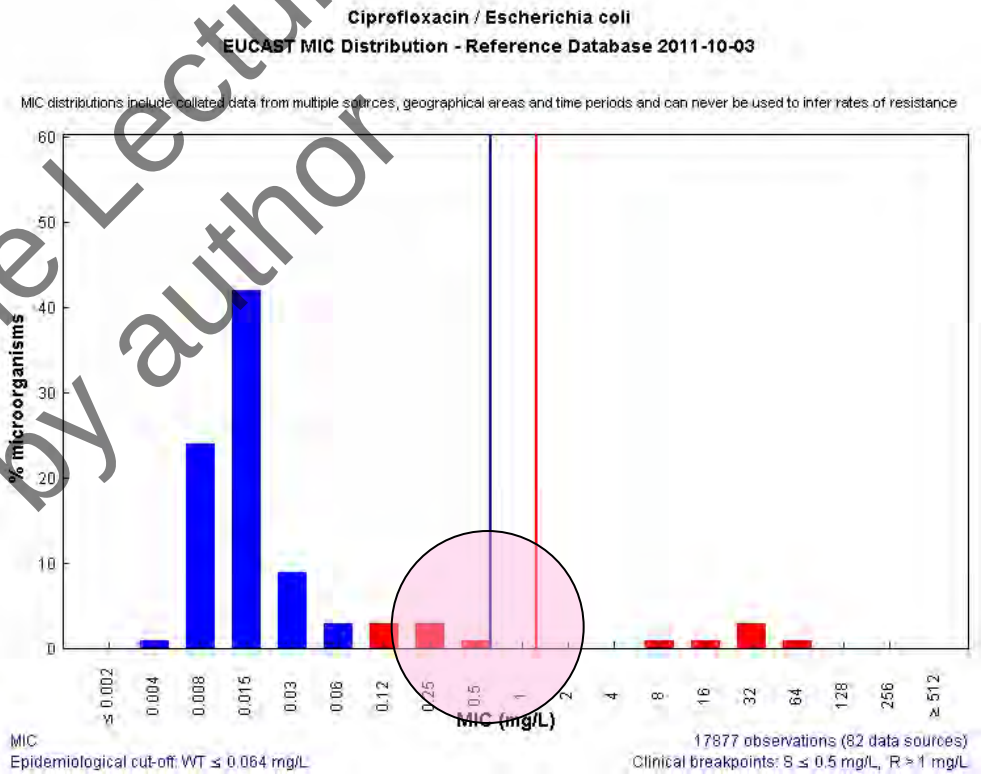
Hakanen A et al. JCM 1999; 37: 3572





Nalidixic acid and ciprofloxacin vs plasmid-mediated quinolone resistance

Resistance mechanism	MIC (mg/L)	
	NAL	**CIP**
WT	2-16	0.004-0.064
QnrA	8-32	0.12-2
QnrB	16	0.25-1
QnrS	8-32	0.12-0.5
QnrD	4-8	0.12-0.25
QnrC	16	0.25
AAC(6')1b-CR	8-16	0.12-0.5
Qep	1-2	0.25
OfxcAB	64	0.12



Can nalidixic acid also fail in case of chromosomal mutations?

Table 2. Characteristics of the 11 *Salmonella enterica* serovar Typhi isolates belonging to subpopulation B, France, 2007–2009

Isolate	Year	Geographic origin	Antimicrobial drug resistance type	Disk diffusion, mm*		MIC, µg/mL		*gyrB*	Haplotype	PFGE
				Nal	Cip	Nal	Cip			
97-5123	1997	Unknown	Cip^{DS}	18 [I]	28 [S]	8 [S/S]	0.125 [S/S]	Tyr464	Non-H58	X8
02-2759	2002	India	Cip^{DS}	19 [I]	26 [S]	4 [S/S]	0.125 [S/S]	Phe464	H58	X2
05-1578	2005	India	Pans susceptible	18 [I]	28 [S]	8 [S/S]	0.047 [S/S]	Asp466	Non-H58	X6
05-2556	2005	India	Cip^{DS}	17 [I]	31 [S]	16 [I/S]	0.19 [S/S]	Phe464	Non-H58	X7
05-9141	2005	India	Cip^{DS}	17 [I]	28 [S]	12 [I/S]	0.125 [S/S]	Tyr464	Non-H58	X3
06-426	2006	India	Cip^{DS}	20 [S]	25 [S]	8 [S/S]	0.125 [S/S]	Tyr464	Non-H58	X3
07-6086	2007	Tunisia	Pan susceptible	16 [I]	31 [S]	16 [I/S]	0.047 [S/S]	WT	ND	ND
08-7675†	2008	India	ASCSulTmpSXTCip^{DS}	18 [I]	28 [S]	8 [S/S]	0.125 [S/S]	Phe464	H58	X1
09-1986†	2008	India	ASCSulTmpSXTCip^{DS}	18 [I]	27 [S]	8 [S/S]	0.125 [S/S]	Phe464	ND	X1
09-0350	2009	Unknown	Cip^{DS}	19 [I]	27 [S]	8 [S/S]	0.125 [S/S]	Phe464	Non-H58	X5
09-2317	2009	French Guyana	Pansusceptible	19 [I]	32 [S]	8 [S/S]	0.032 [S/S]	Glu468	Non-H58	X4

*PFGE, pulsed-field gel electrophoresis; Nal, nalidixic acid; Cip, ciprofloxacin; ND, not determined; WT, wild type; A, ampicillin; S, streptomycin, C, chloramphenicol; Su, sulfamethoxazole; Tmp, trimethoprim; SXT, cotrimoxazole; Cip^{DS}, decreased susceptibility to ciprofloxacin. Disk diffusion test was performed and interpreted ([S], susceptible; [I], intermediate) following recommendations of antibiogram committee of the French Society for Microbiology (CA-SFM); MICs were determined by Etest strips and categorization was made according to CA-SFM and Clinical and Laboratory Standards Institute. †Previously described same patient (*12*).

Accaou-Demartin M. EID 2011

FQ-R Salmonellae vs MIC ciprofloxacin

	MIC ciprofloxacin											
type of resistance	≤0,008	0,016	0,032	0,063	0,125	0,25	0,38	0,5	0,75	1	2	4
83TAC					2	1						
83TAC+efflux								1		1		
83-TTC					2	2						
83-TTC+efflux												1
87-GGC					12							
87-GGC+efflux						1						
87-TAC				1	16	4		4				
87-AAC					22	4		3				
87-AAC+efflux										1		
87-TAC+aac6'										1		
efflux					1							
parC					1	1						
qnrB						4	1	5		1		
qnrS					3	11		32	3	3	1	
aac6'					1							
qnrS+aac6'								1				
qnrA						1						
No mechanism	3	79	31	9	1							

Courtesy of Robert Skov/Niels Frimodt Møller

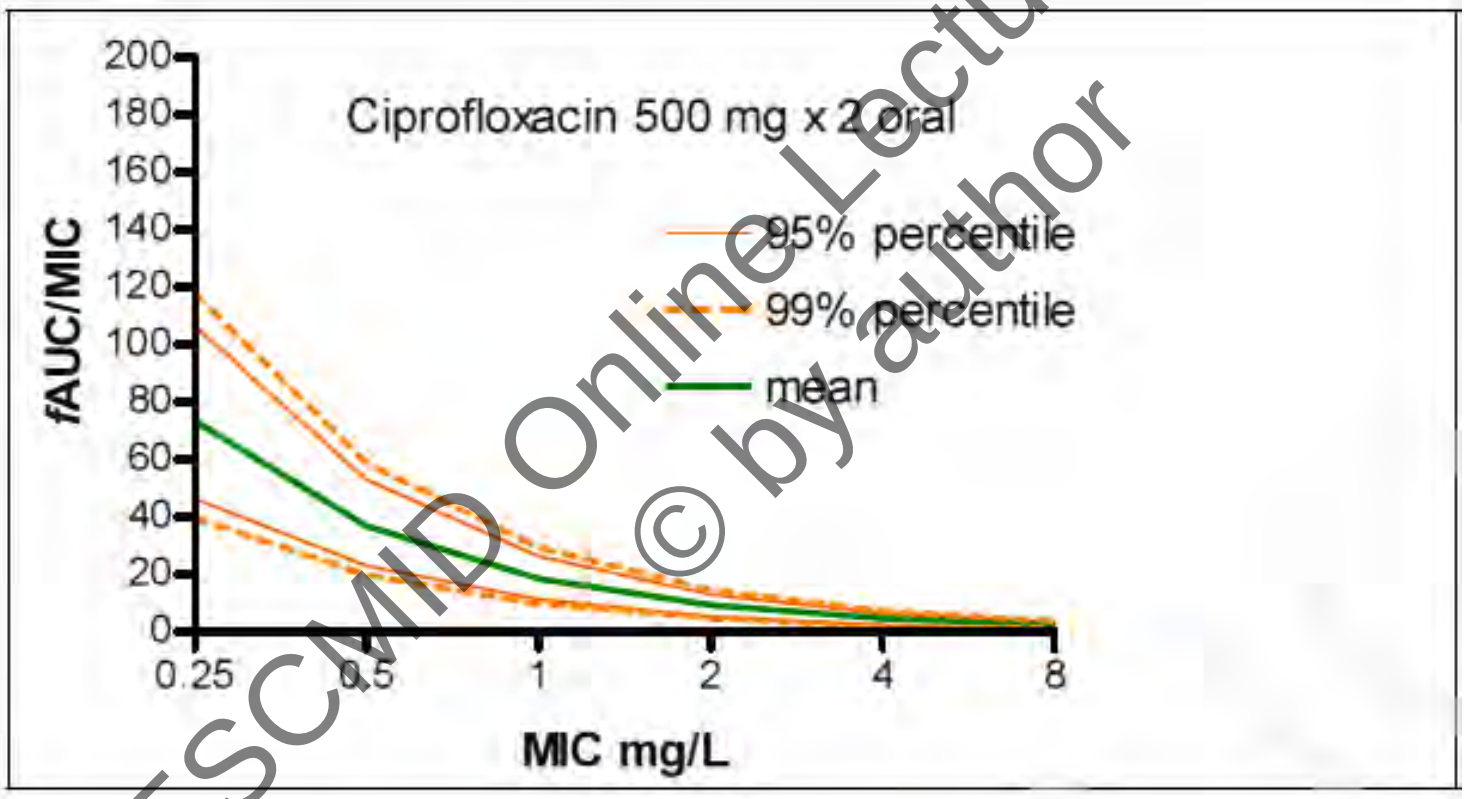
Treatment failure in Salmonella related to CIP MIC

Parameter	Susceptibility to CIP		Significance
	CIP<0.12	**CIP 0.12-1**	
Antimicrobial-related fever clearance (h)	72	92	P=0.01
Ciprofloxacin-related fever clearance	64	90	p=0.153
Treatment failure	2/46	4/24	RR 2.5, 95% CI 1.2-5.1

Crump JA et al. AAC 2008; 52: 1278

Still no similar published data in *E. coli*

PK/PD in support of higher breakpoint than CIP 0.06 mg/L in non-Salmonellae (?)



www.eucast.org (rationale document ciprofloxacin)

Decision of EUCAST: ECOFF for *Salmonella* spp, PK/PD for other Enterobacteriaceae

Fluoroquinolones	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		Notes Numbers for comments on MIC breakpoints Letters for comments on disk diffusion
	S ≤	R >		S ≥	R <	
Ciprofloxacin[1]	0.5	1	5	22	19	1. *Salmonella* spp. - there is clinical evidence for ciprofloxacin to indicate a poor response in systemic infections caused by *Salmonella spp.* with low-level fluoroquinolone resistance (MIC>0.064 mg/L). The available data relate mainly to *S. typhi* but there are also case reports of poor response with other *Salmonella* species.
Levofloxacin	1	2	5	22	19	
Moxifloxacin	0.5	1	5	20	17	
Nalidixic acid (screen)	NA	NA		NA	NA	

Is it possible to develop a disk test for Salmonella corresponding to CIP ECOFF?

	≤0,008	0,016	0,032	0,063	0,125	0,25	0,5	1	2	4	Total
16									1		1
18								1			1
21							1			1	2
22							4	2			6
23					1	3	14	2			20
24					1	2	11	5			19
25					2	6	8				16
26					2	8	3				13
27					13	3	4				20
28					16	4	2				22
29		1			17	2					20
30			1	1	5	1					8
31		2			4						6
32		3	4	2							9
33		5	3	2							10
34		7	1								8
35	1	3	1								5
36		9	3								12
37		12	4								16
38		10	4	1							15
39	2	3									5
40		3									3
Total	3	58	21	6	61	29	47	10	1	1	247

- Suggests cut-off at 31 mm for 5 µg disk
- Courtesy of Robert Skov/Niels Frimodt Møller
- Works in a reference lab, but…

Should we search for PMQR in non-Salmonella Enterobacteriaceae?

- [x] Yes, of course
- [x] Don't know
- [x] Maybe, but too cumbersome
- [ ] What is PMQR?

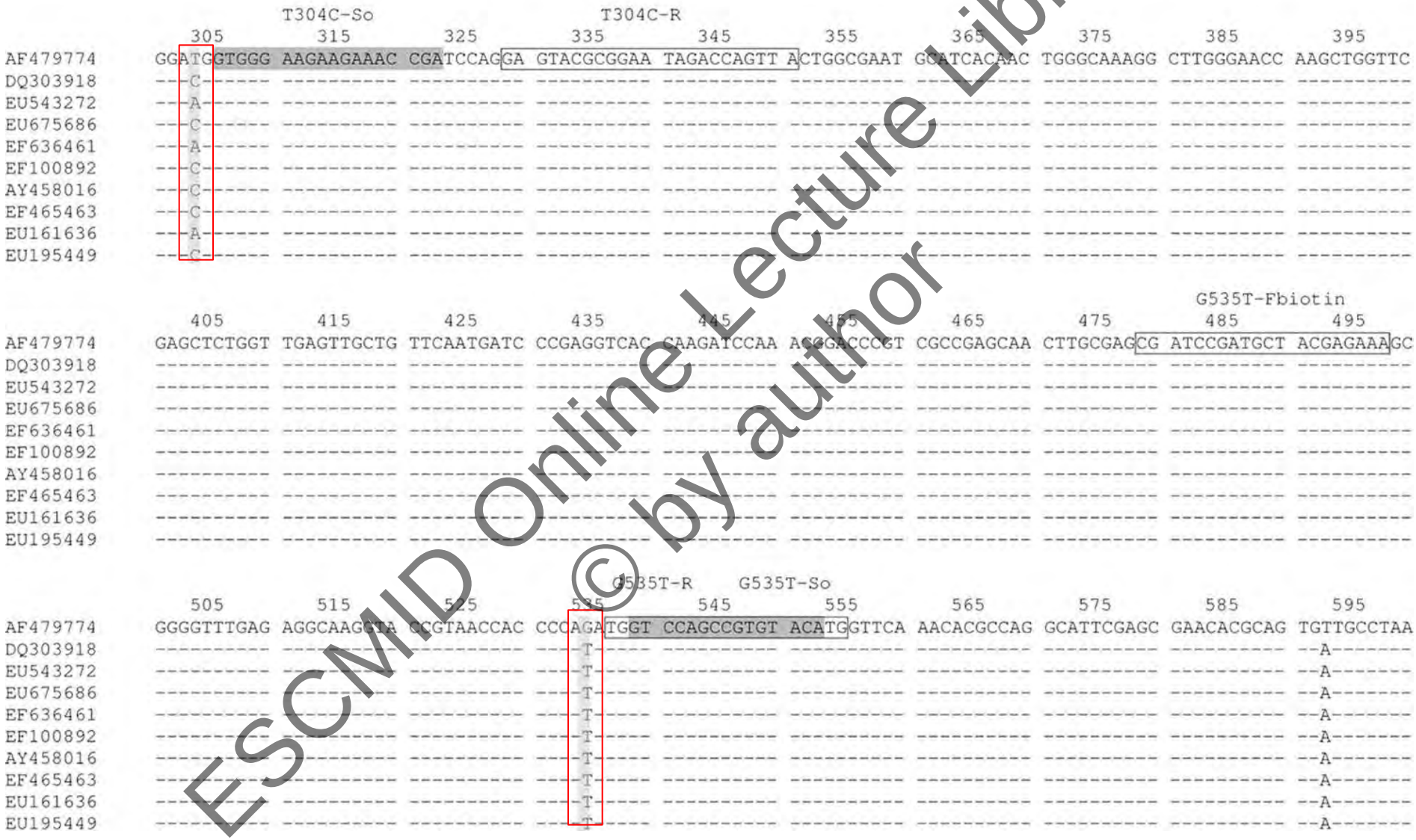
How to detect FQ-resistance with genotypical methods

- Chromosomal resistance: sequencing should be preferred – usually of both *gyrA* and *parC*
- Efflux: quantification of mRNA for efflux pumps

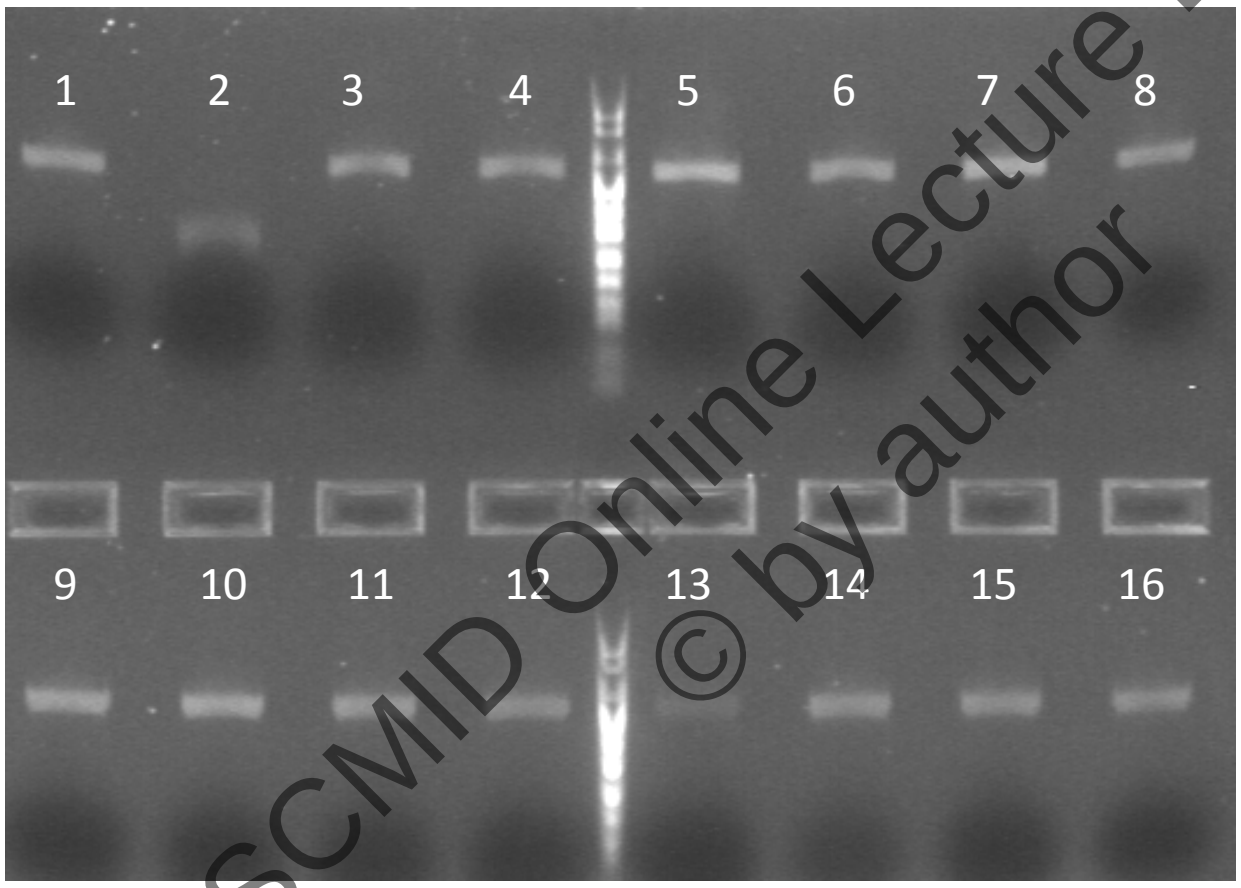
- In both cases such resistance is easy to identify with phenotypical methods

- Therefore: PMQR is the most interesting type of FQ-resistance to investigate with genotypical methods

AAC6(')1b-CR



AAC6(')1b-CR – digestion with *BtsCI*

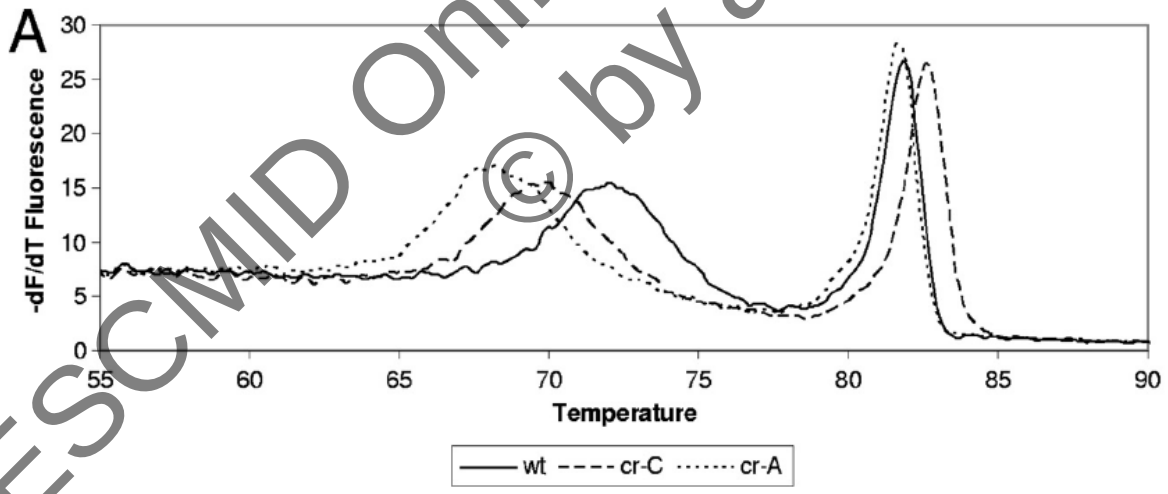


1. Amplify
2. Cut (5 µL template + 1 µL enzyme + 2 µL buffer + 12 µL H_2O)
3. 2 h digestion at 50 °C
4. Run out on gel

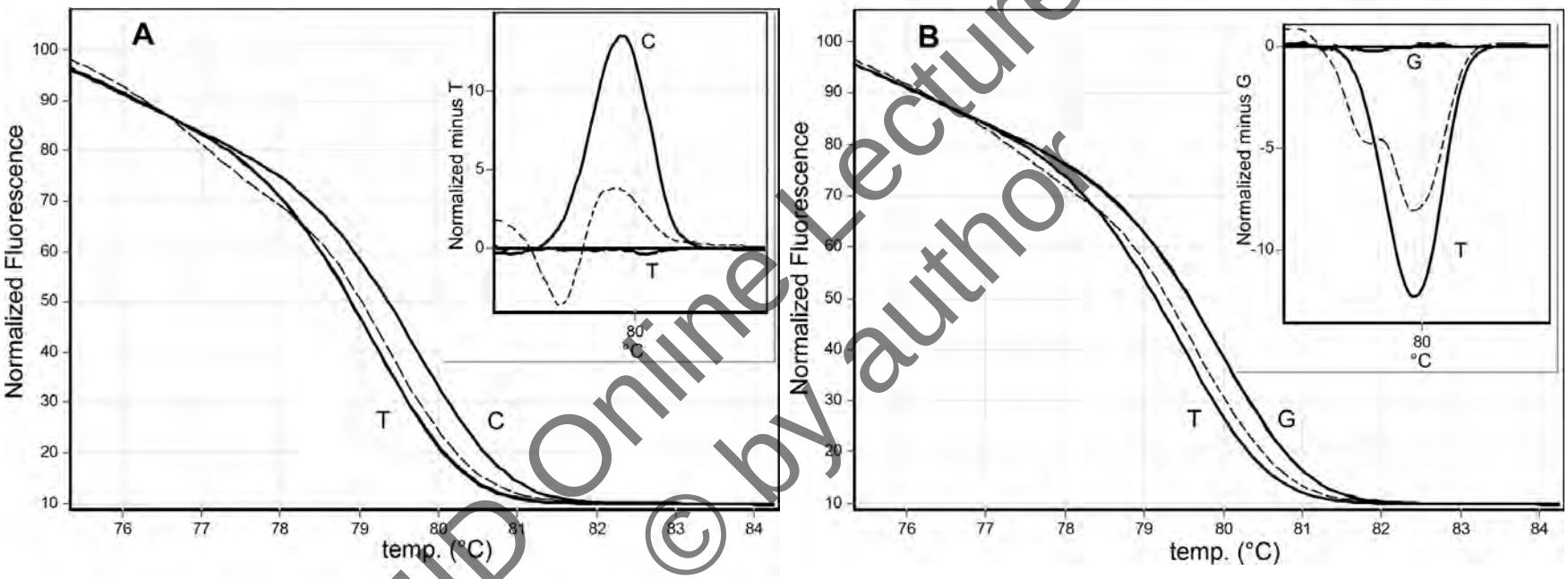
Lane 1. Positive control for aac(6´)-Ib-cr,
Lane 2. Negative control for aac(6´)-Ib-cr (which is wild type aac(6´)-Ib),
Lane 3 to Lane 16 all E.coli are CR variants of aac(6´)-Ib.

AAC6(')1b-CR – other options

- Sanger sequencing
- Pyrosequencing (Guillard T et al. JCM 2010; 48: 286-289)
- High-resolution melting point analysis (real-time PCR) (Bell JM et al. AAC 2010; 54: 1374-1380
- Nt 304 T→C (easy to detect) or T→A (similar melting point)
- Probe binding to the WT included

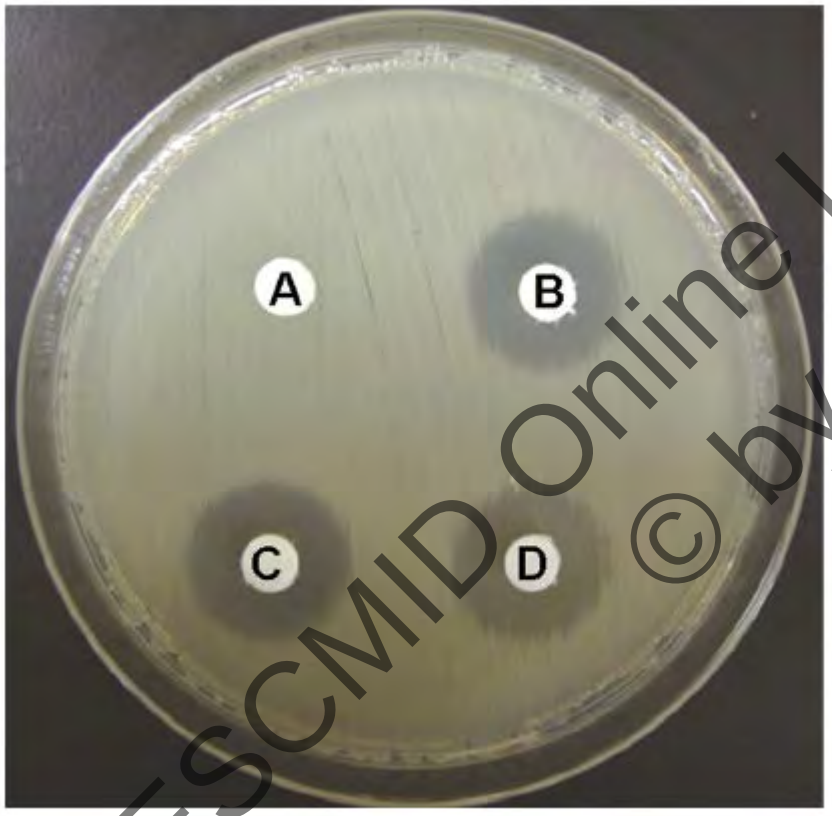


AAC6(')1b-CR – HRM and two targets



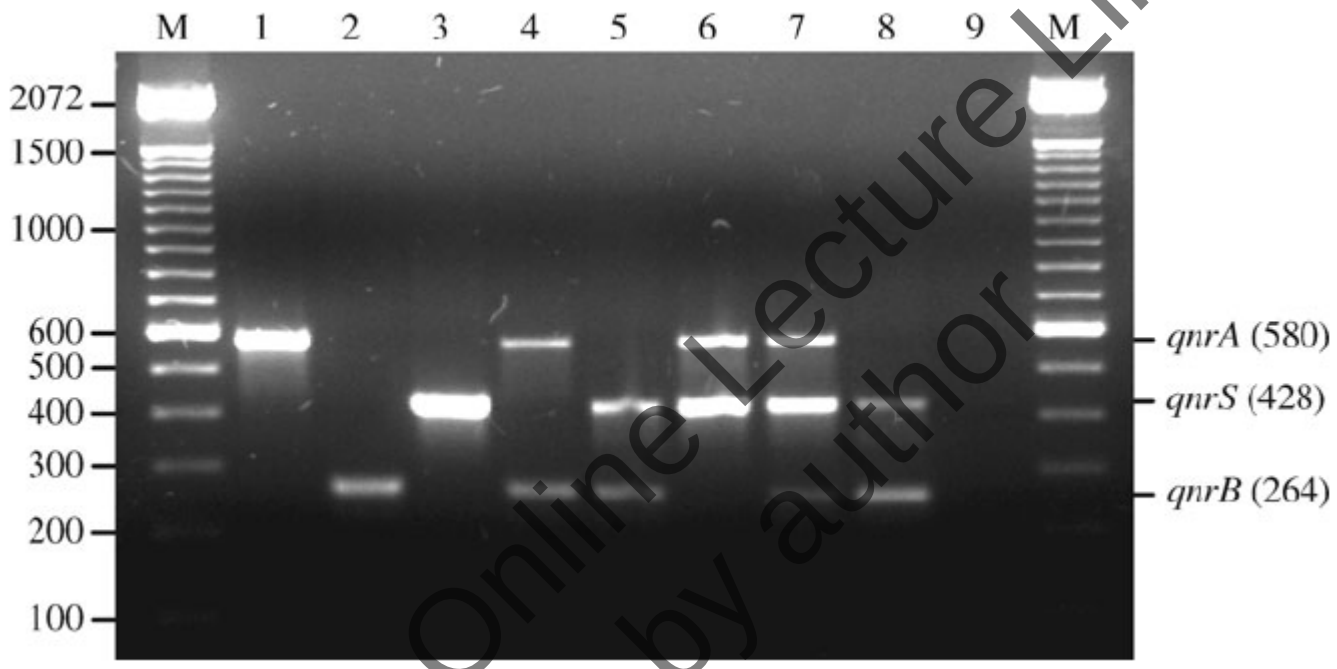
- Analyzing both *aac6*(')1b-CR characterizing regions
- Hidalgo-Grass C and Strahilevitz J. AAC 2010; 54: 3509

Phenotypic method for identifying AAC6(')1b-CR



- Colonies grown in LB broth containing norfloxacin 8 mg/L
- After 18 h 10 µL was transferred to blank disks on a plate inoculated with *E. coli* ATCC 25922
- Additionally 80 ng of norfloxacin was added to all disks (10 µL of LB without bacteria)
- Inhibition zone decrease of >10 mm was indicative of presence of the CR-variant
- Wachino JI. JCM 2011; 49: 2378

Qnr – classical multiplex PCR



- Cattoir V et al. JAC 2007; 60: 394
- Limitations:
  - → Not adapted to real-time PCR
  - → Does not cover *qnrC* and *qnrD*
  - → Challenging to detect *qnrB*-variants due to high diversity

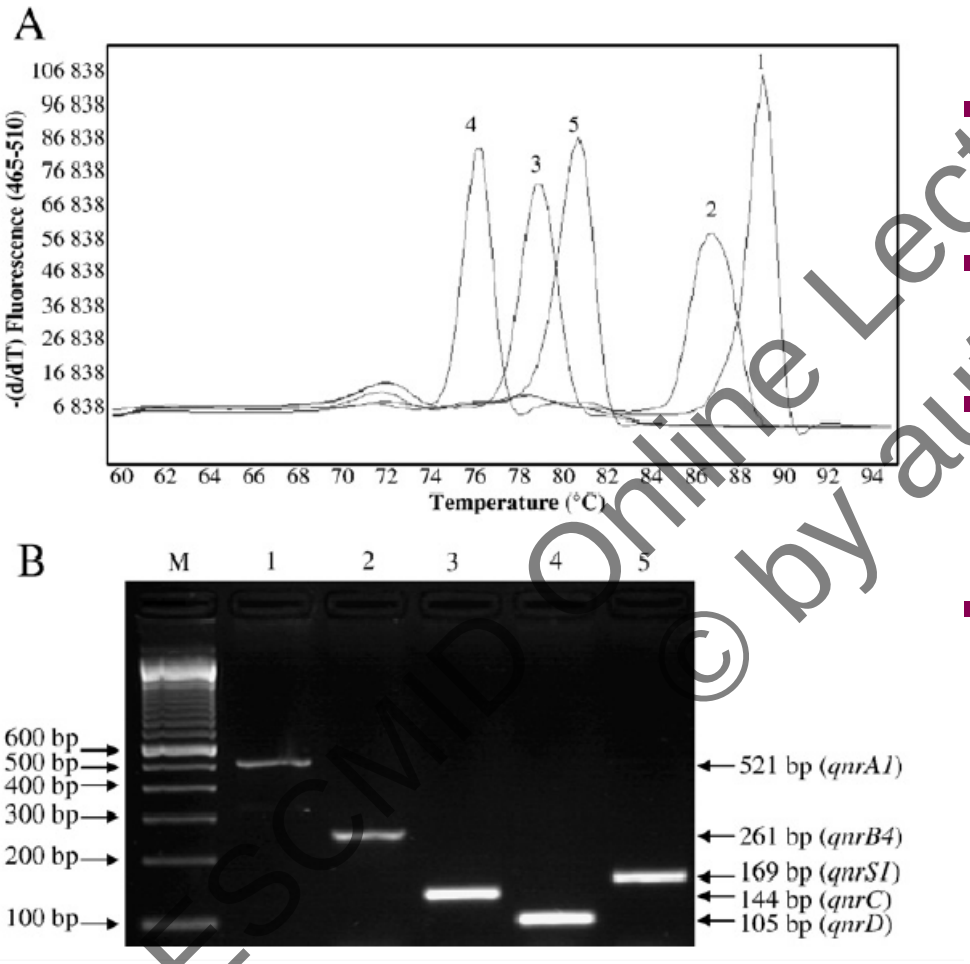
QepA

- E.g. Yamane K. AAC 2008; 52: 1564
- Amplification of a 199 bp fragment
- Well suited both for real-time PCR and classical gel PCR
- Other options (403 bp fragment): Minarini LAR et al. JAC 2008; 62: 474

OfxcAB

- PCR targeting *ofxcA* and *ofxcB* has been used (products of 392 and 512 bp, respectively)
- Kim HB. AAC 2009; 53: 3582
- No multiplex-PCR with other PMQR-variants is currently available

Simultaneous detection of Qnr and QepA

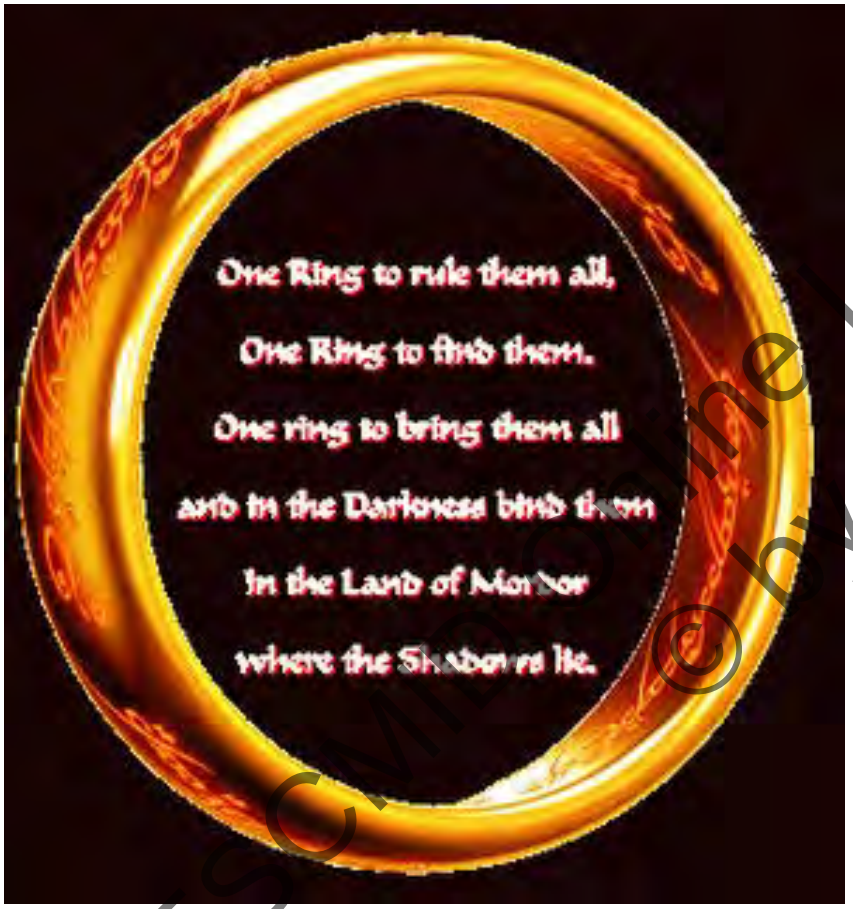


- High-resolution melting supermix (Roche)
- Multiplex-PCR for all *qnr*-genes
- Simplex SYBR-Green assay for *qepA1 and qepA2* (another temperature profile)
- Guillard T et al. DMID 2011; 70: 253

Detection of chromosomal resistance

- *gyrA*: amino acid 67-106
- *parC*: amino acid 78-84
- Efflux:
  - → Phenotypic assays with efflux inhibitors PAβN or reserpine
  - → Quantification of mRNA for Enterobacteriaceae (AcrAB-TolC; Swick MC AAC 2011; 55: 921) or for e.g. *S. pneumoniae* (PmrA, PatA, PatB; Garvey MI et al. AAC 2011; 55:190)

One quinolone to rule them all….



Gram-positives	• Yes • Norfloxacin
Gram-negatives	• No • But we used to…

Key messages

- Chromosomal resistance is usually not challenging to identify with phenotypical methods
- Such resistance can also be studied with sequencing, phenotypic inhibition assays or quantification of mRNA
- The above activities are mainly meaningful for research purposes
- Still debated whether it is clinically important to detect PMQR
- PMQR are non-wild-type, and hence EUCAST ECOFFs may be used to identify isolates with putative presence of PMQR (disk test is being validated)
- A number of convenient molecular assays available for screening, but an array would be good for the future