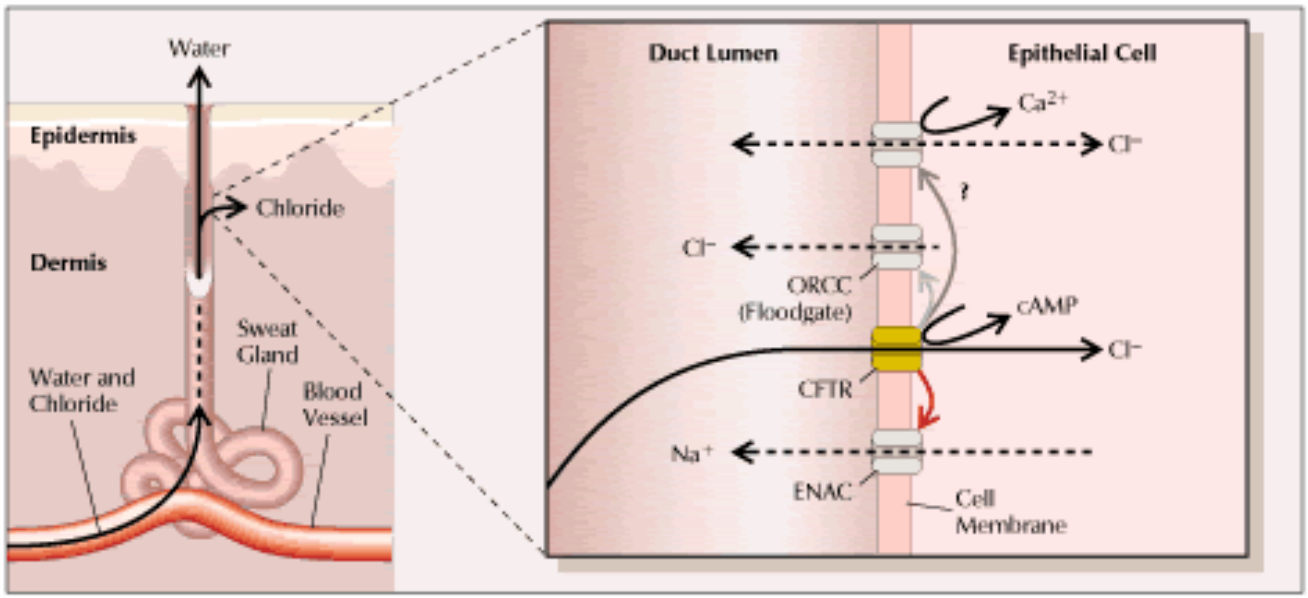
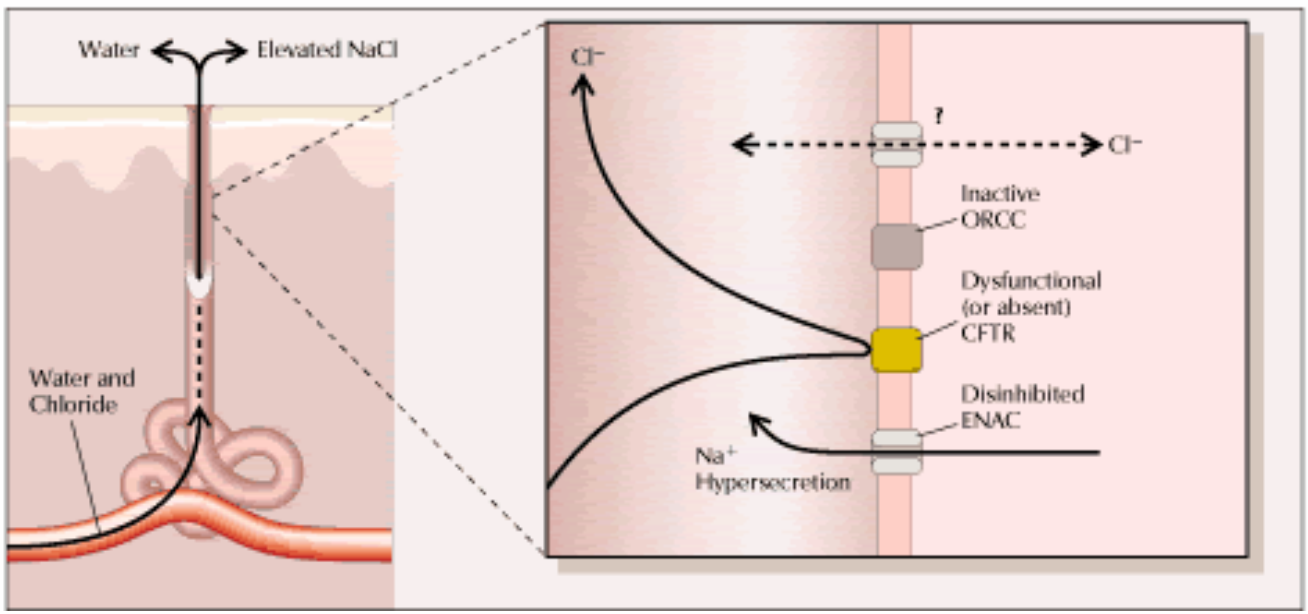
Phénotypes et génotypes de résistance à l'oxacilline de Staphylocoques dorés isolés chez des patients mucoviscidosiques belges

Dr Anne Vergison
Département des maladies infectieuses
Unité d'Epidémiologie et d'hygiène hospitalière
ULB-Huderf

Anomalie de canal chlore (CFTR) dans la mucoviscidose



Normal



Muco

Table 2. Host abnormalities that predispose CF patients to chronic lung infections

Abnormality	Proposed impact	Proposed intervention(s)
Abnormal cystic fibrosis transmembrane regulator (CFTR)	Altered secretions (low volume of airway surface fluid and hypertonicity) leads to thick dehydrated mucus, impairment of mucociliary escalator, and impaired defensin-mediated antimicrobial activity	• Chest physiotherapy • Inhaled DNase • Gene therapy* • Alter electrolyte and water balance by aerosolized amiloride (block Na^+ uptake)*, uridine triphosphate (UTP) (increase Cl^- efflux),* or provide novel peptides†
Increased expression of asialoganglioside (aGM1)	Increased *P. aeruginosa* and *S. aureus* binding to respiratory epithelial cells	• Anti-aGM1 blocking antibody†
Defective CFTR-mediated uptake of *P. aeruginosa* by respiratory epithelial cells	Decreased clearance of internalized *P. aeruginosa* with sloughed epithelial cells	• Gene therapy*
Abnormal regulation of pro-inflammatory cytokines	• Hyperexuberant neutrophil recruitment and release of neutrophil oxidants	• Anti-inflammatory therapy, eg, steroids or ibuprofen beneficial, but associated with side effects such as cataracts, poor growth, or gastrointestinal bleeding
Increased IL-8 expression and TNF-alpha	• Upregulation of human mucin genes	• More selective anti-inflammatory agents†
Variant mannose-binding lectin (MBL)	Polymorphisms may differentially bind bacterial surface carbohydrates	• MBL replacement†

*Investigational intervention.
†Theoretic intervention.

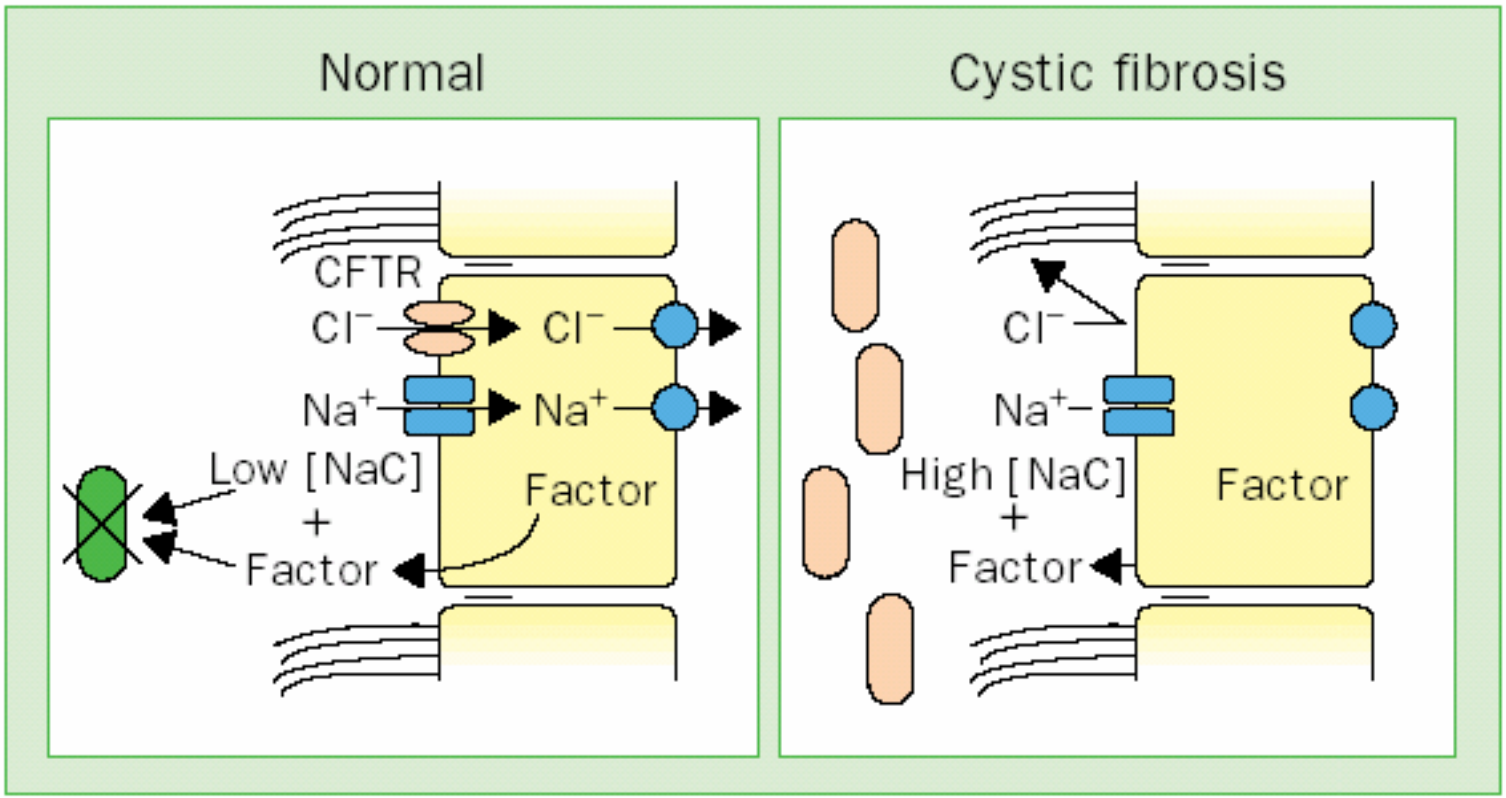
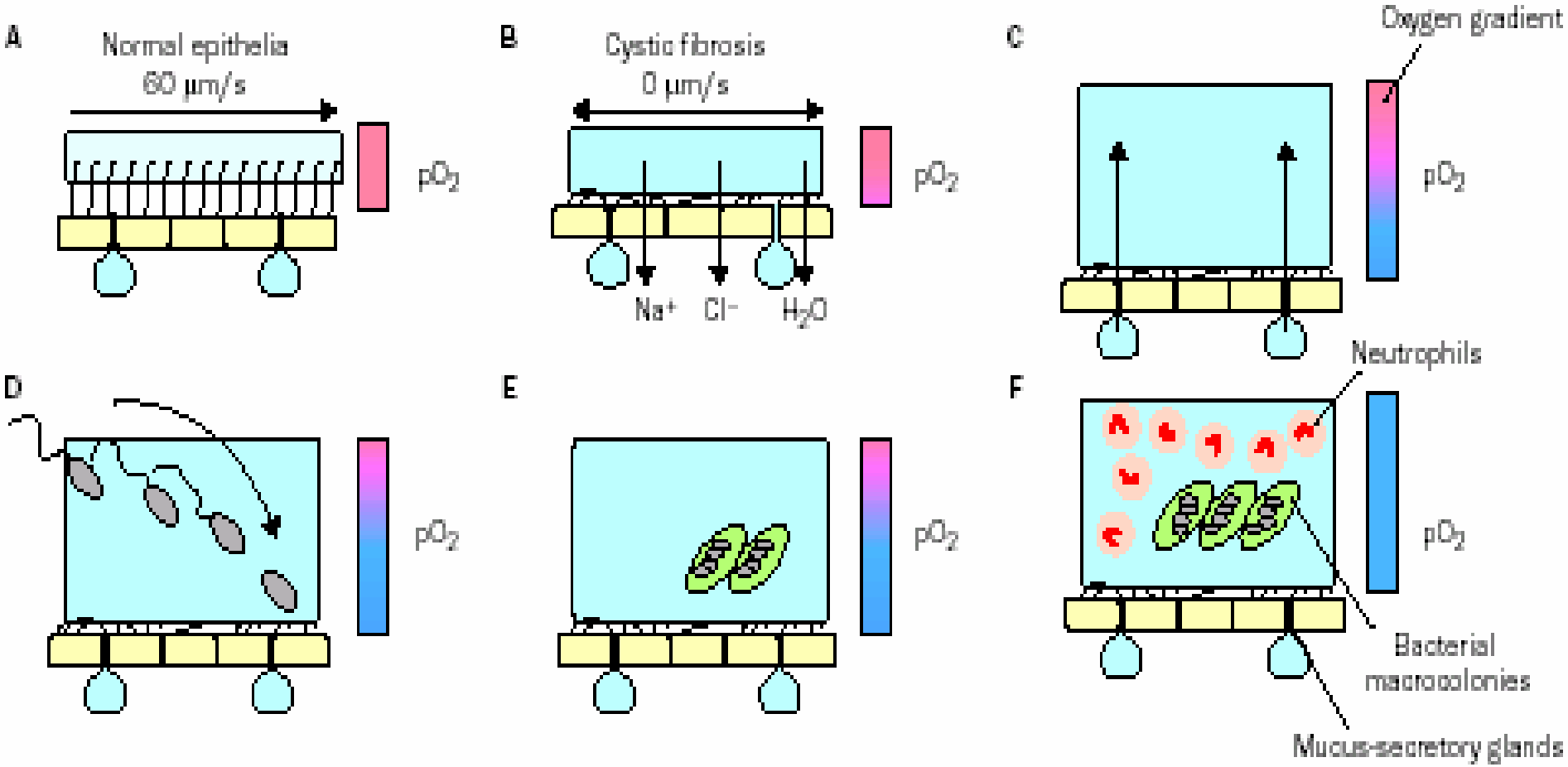


Figure 2: **Hypothesis arguing that CF respiratory epithelia fail to kill bacteria because of inactivation of host defensins by raised salt concentration in airway surface fluid**
(Figure courtesy of Dr Michael J Welsh, and the Cystic Fibrosis Foundation, Bethesda, MD, USA.)

Rosenstein, Lancet 1998

Hypothèse mucus déshydraté isotonique dans le poumon de CF



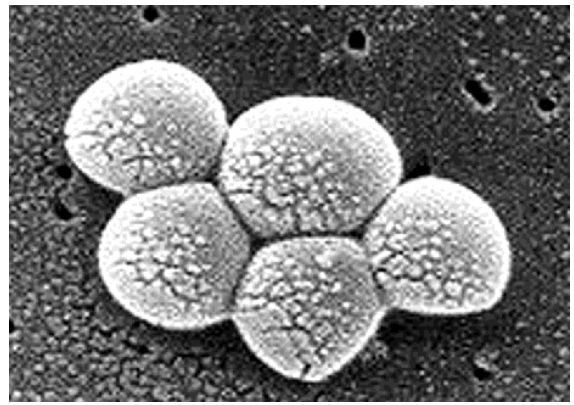
Ratjen, Lancet, 2003

Poumon de muco

Pathophysiologie: *S.aureus*

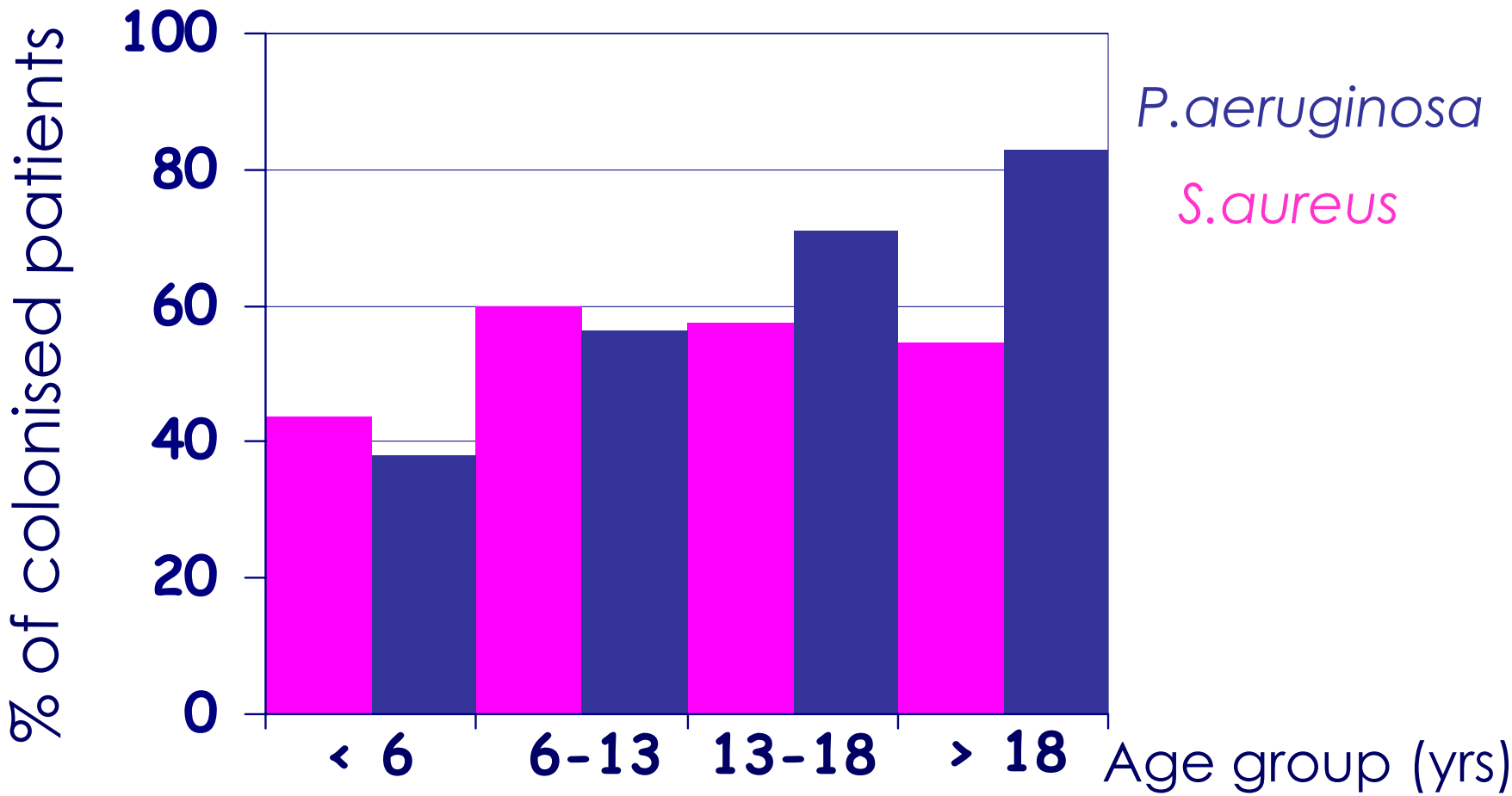
- Liaison aux mucines respiratoires
- Haute affinité pour asialoganglioside 1dont la quantité est augmentée chez muco

S. aureus



- Common pathogen in CF patients
- Prevalence from 44% to 60% in CF in Europe

S.aureus colonisation in CF patients



From G.Mastella, European Registry for Cystic Fibrosis data 1994-1999

Prévalence des différents pathogènes selon l'âge

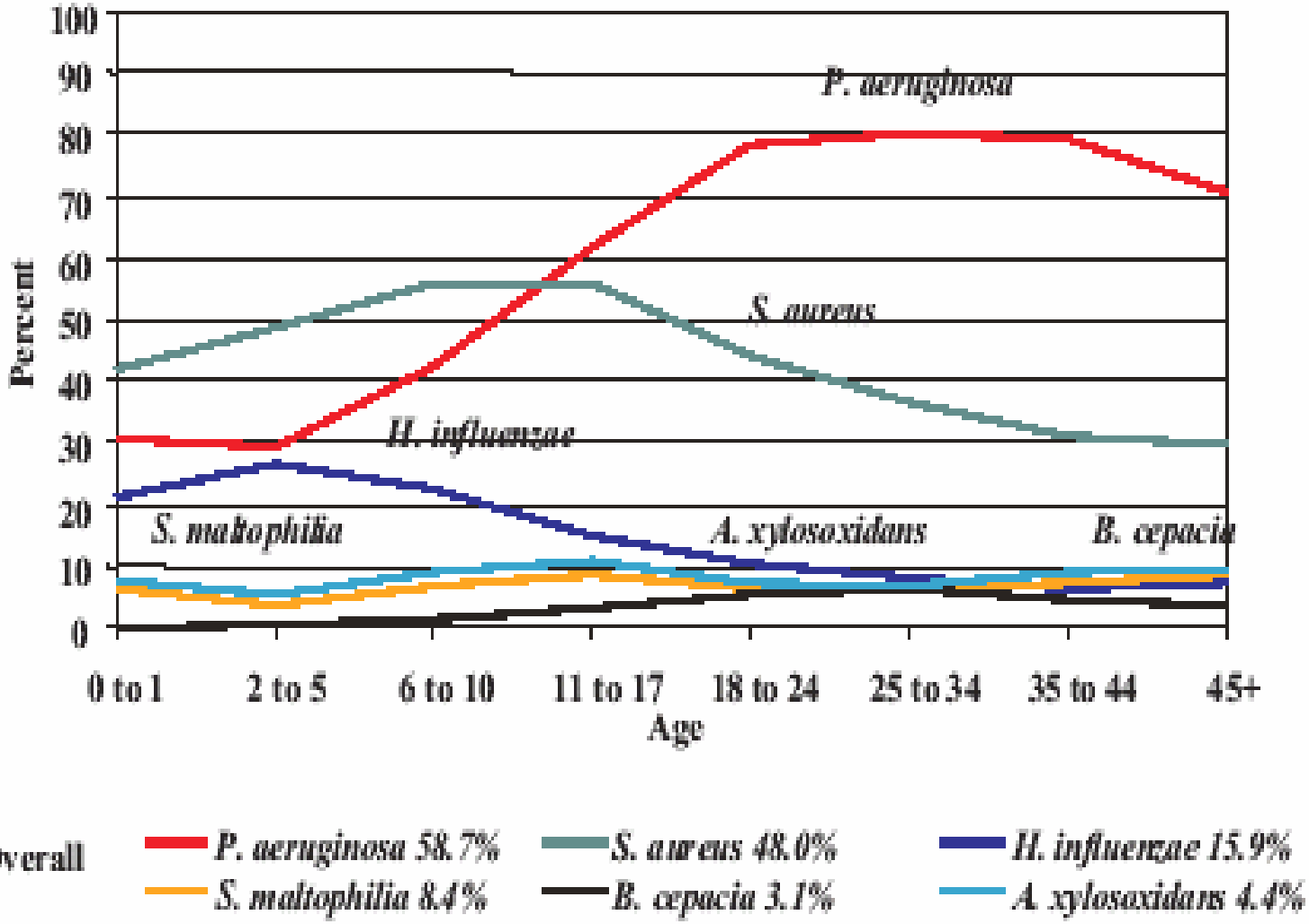


Fig. Age-specific prevalence of respiratory pathogens in CF patients: CFF National Patient Registry Data 2001.[9]

S.aureus in CF

- Colonisation more frequent in CF
- Chronic broncho-pulmonary infections
- Clinical problems mainly in < 10 yrs
- Before AB, 90% of CF patients died of *S.aureus* infection
- Interaction with other pathogens in CF lung?

Methicillin resistant *S. aureus* (MRSA)

- Increasing prevalence in the pediatric population
- Important infection control issue in hospitals
- Emergence of glycopeptide resistance (VISA & VRSA)

MRSA in CF

- Prevalence up to 23% in US CF (2001) (higher in inpatients)
- Probably nosocomially acquired
- Clinical impact ???
  - Very few data
  - Pediatric case-control study:
    - ↑ IV courses in MRSA
    - Worse chest X-ray in MRSA
    - No difference in nutritional status or lung function

Miall, Arch Dis Child, 2000

Small Colony variants (SCV) of *S. aureus*

- Recurrent and persistent infections
- Intracellular (in CF lung?) => escape host defenses and AB
- Less tissular damage, latent state, but reversible
- More resistant to AB and difficult to eradicate
- Can be epidemic (MRSA)
- Auxotrophs

« Normal » *S. aureus* strains

24 h Blood agar

SCV *S. aureus* strain

SCV 48H blood agar
« fried egg » appearance

SCV and CF

- USA: Gilligan; 1987: 95 *S.aureus*: 21% thy$^-$ SCVs
- Germany: Kahl; 1998: 53 *S.aureus*: 49.1% SCVs
- Poland: Sadowska; 2002: 63 *S.aureus* from CF children: 31,7% SCV
- Belgium 2 case reports of CF patients with SCVs
  - VISA, haemin auxotroph — Denis; JAC, 2002
  - Oxa R with abnormal PBP 3 — Pinho; ICAAC, 1999

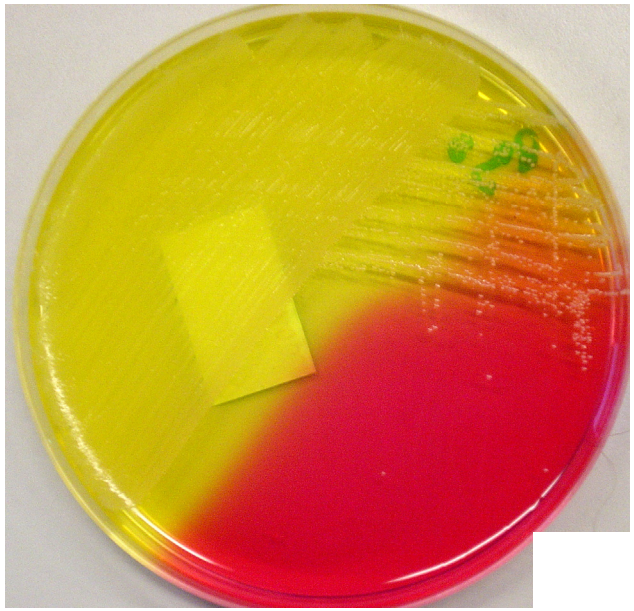
Microbiology in CF, L. Saiman and CF Foundation conscensus conference, AJIC May 2003

Table 3. Recommended media and processing for recovery of CF pathogens

Organism	Recommended media or processing*
S. aureus	Mannitol salt agar
	Columbia/colistin-nalidixic acid agar
H. influenzae	Horse blood or chocolate agar (supplemented or not with 300 mg/L bacitracin) incubated anaerobically
P. aeruginosa	MacConkey agar
B. cepacia complex	OFPBL agar, PC agar, BCSA
S. maltophilia	MacConkey agar, VIA agar
	DNase agar confirmatory media or biochemical or molecular identification
A. xylosoxidans	MacConkey agar
	Biochemical identification assay
Mycobacterial spp.	NALC-NaOH and oxalic acid decontamination step
Aspergillus spp.	*Aspergillus* spp. and other molds do not grow well on Mycosel, but do grow well (though not selectively) on other media used for CF specimens, especially OFPBL
Other gram-positive organisms	Sheep blood agar supplemented with neomycin and gentamicin (streptococcal selective agar)
Other gram-negative organisms	MacConkey agar

*Detection of some pathogens may be enhanced by prolonging incubation for as long as 4 days to allow slow-growing colonies to become apparent. All media are commercially available.

SCV *S.aureus* strains



SCV 48h MSA, Schaedler and blood agar

Oxacillin Resistance in *S. aureus*

- *mecA* → PBP2a
  - Several factors modulating resistance
    - Internal factors (peptidoglycan configuration and synthesis regulation,…)
    - External factors (salt concentration, temperature, …)
- Penicillinase hyperproduction
- Altered PBPs

Detection of oxacillin Resistance in *S. aureus*

TABLE 1. Sensitivities of the recommended oxacillin methods and of the new methods for detection of 83 MRSA clinical isolates

Test	Sensitivity[a]		
	Overall	For class 1 MRSA	For other MRSA classes
Agar screen with 6 μg of oxacillin ml^{-1}	94	92.3	94.7
E-test for oxacillin MICs	91.6	73.1	100
Disk diffusion			
Oxacillin, 5 μg[b]	95.2	84.6	100
Oxacillin, 1 μg[b]	96.4	88.5	100
Cefoxitin, 30 μg[c]	100		
Moxalactam, 30 μg[c]	100		
Vitek 2	94	92.3	94.7
MRSA-screen	97.6	100	96.5

[a] Percentage of the 83 *mecA*-positive isolates for which positive test results were obtained.

[b] Inoculum, 10^8 CFU ml^{-1}. Incubation was carried out for 18 h at 37°C.

[c] Inoculum, 10^6 CFU ml^{-1}. Incubation was carried out for 18 h at 37°C.

Felten, JCM 2002

CFF 2003 recommandation

- Oxacillin agar for MRSA
- AB susceptibility testing: agar based diffusion assays (disks or E-tests)
- Commercial automated microbroth dilution assays: unacceptebly high rates of errors (*P.aeruginosa*)

L. Saiman AJIC 2003

Detection of oxacillin Resistance in *S. aureus* SCVs

TABLE 1. Comparison of different methods for detection of methicillin resistance in SCVs

Strain	Auxotrophy[a]	Agglutination of PBP 2a[b]		Vitek 2 MIC (μg/ml)[c]	Disk diffusion[d,e]		Etest[e] (μg/ml)		Microdilution (μg/ml)	*mecA* PCR result[b]
		1–2 colonies	100–200 colonies[f]		24 h	48 h	24 h	48 h		
1	M	−	+	MC	NG	NG	NG	NG	>32	+
2	H	+	+	>8	NG	R	NG	>256	>32	+
3	H	−	+	>8	R	R	>256	>256	>32	+
4	H	+	+	>8	S	S	0.064	0.064	0.25	+
5	H	−	+	>8	R	R	>256	>256	>32	+
6a	H	+	+	<0.5	S	S	0.064	0.064	0.5	+
6b	M, H, T	−	+	>8	S	S	2	3	>32	+
7	H	−	+	MC	NG	NG	NG	NG	>32	+
8	H	+	+	>8	NG	R	NG	>256	>32	+
9	M, T	−	+	MC	NG	NG	NG	NG	>32	+
10	H	−	+	MC	NG	NG	NG	NG	>32	+

Kipp, JCM 2004

2001 Belgium: national study Microbiology of *S. aureus* in Cystic Fibrosis (CF) patients

Objectives

To study

- The prevalence of *S.aureus* colonisation in the Belgian CF patient population
- The prevalence and characteristics of MRSA and SCV of *S.aureus*
- Antibiotic resistance in these *S.aureus* strain

Methods

- Prospective 7 month survey in 2001
- 8 CF clinics and 1 revalidation centre
- *S.aureus* collection: all non-duplicate isolates with distinct phenotype per patient

Definitions

- MRSA: *mecA* gene in a *S.aureus* strain
- Small Colony Variant strains
  - small slowly growing colonies
  - non-pigmented
  - non-haemolytic
  - auxotroph (thymidine, haemin or menadione)

Characterisation of *S.aureus*

- Culture techniques: local procedures in each centre
- Phenotypic identification
  - Coagulase & Pastorex Plus
  - Oxacillin Screen Agar (BBL)
- Genotypic identification: PCR for 16S rRNA, *nuc*, *mec*A genes
- Genomic macrorestriction (*Sma*I) – PFGE typing
- Auxotrophism studies (thymidine, haemin & menadione)

Antibiotic susceptibility testing

- Broth microdilution MIC (NCCLS)
  - Oxacillin
  - Vancomycin
  - Teicoplanin
  - Gentamicin
  - Amikacin
  - Ciprofloxacin
  - Cotrimoxazole
  - Linezolid
- MH broth for normal phenotypes
- MH + haemin, thymidine and menadione for SCV

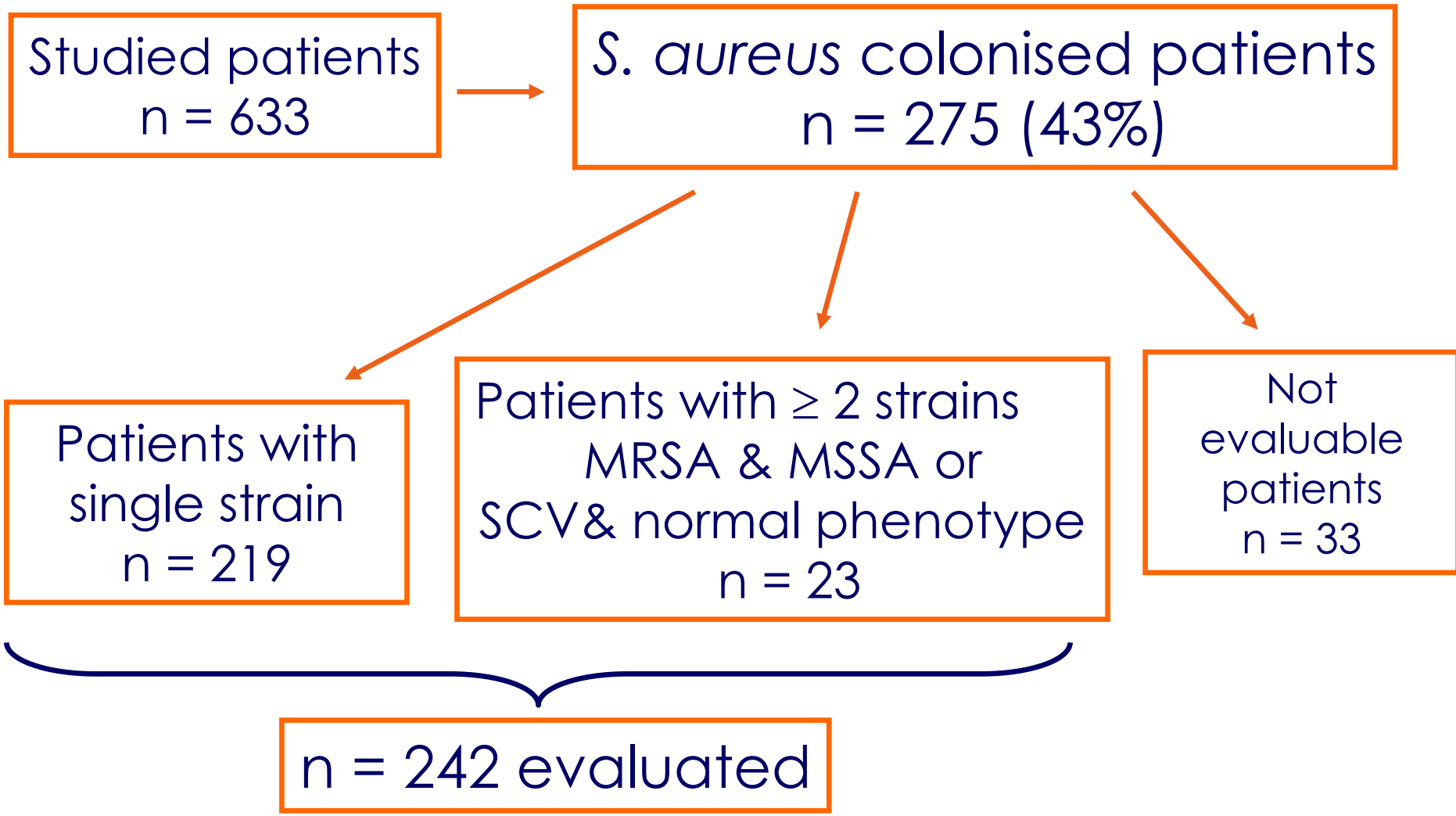
Oxacillin resistance testing

- Broth microdilution MIC (NCCLS)
- MH broth for normal phenotypes
- MH + haemin (1 mg/l), thymidine (100 mg/l) and menadione (1 mg/l) for SCV
- + 2 % NaCl
- Incubation 24h to 48h (SCVs) at 35°C, normal atmosphere

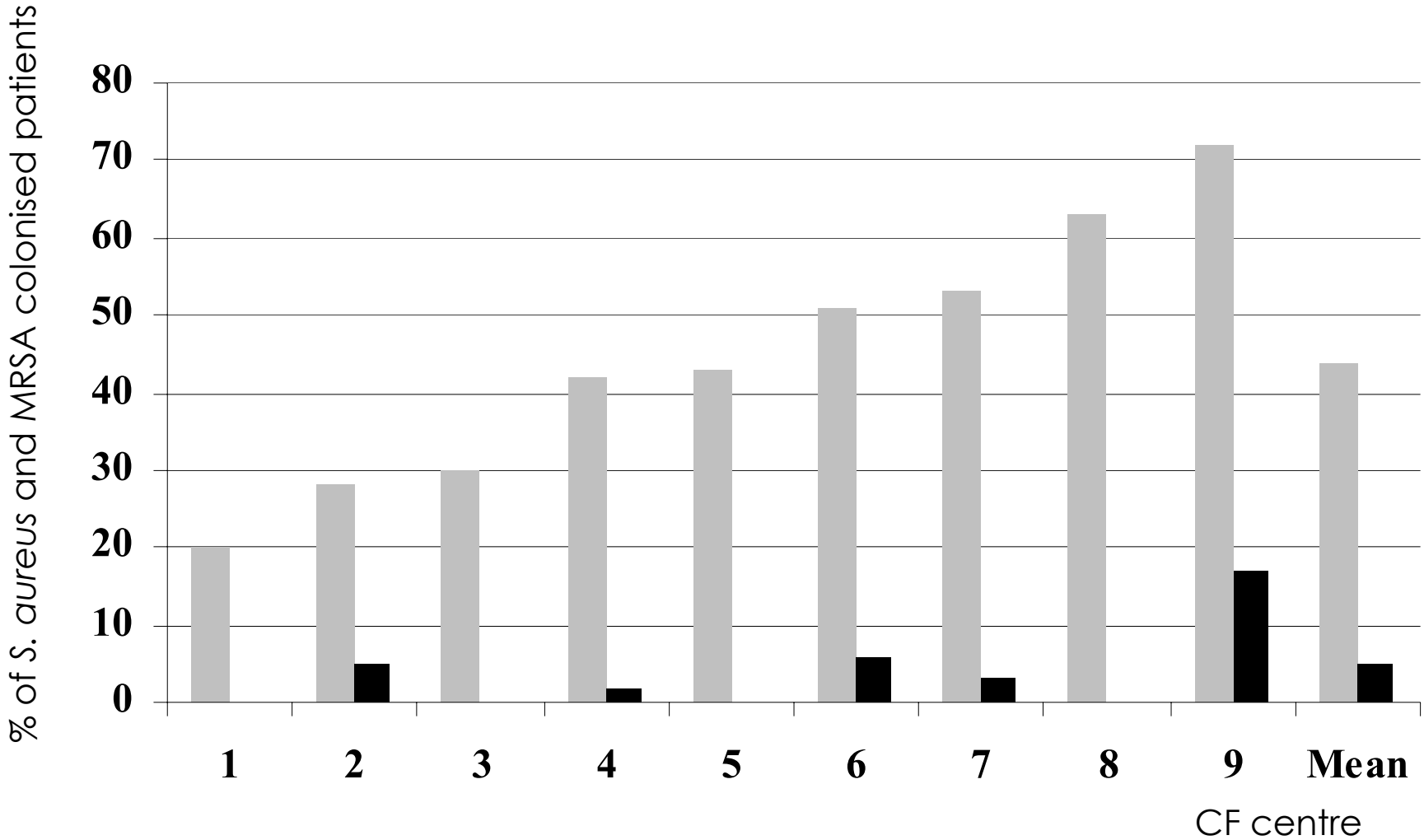
Oxacillin resistance testing of *mecA* negative strains with oxacillin MIC ≥ 4 mg / l

- Broth microdilution MIC
- E-test (AB biodisk)
  - MH agar ± vitamins ± 2% NaCl
  - MH blood agar with and without NaCl
  - Oxacillin
  - Cefoxitin

Results: Prevalence of *S. aureus* colonisation



S. aureus and MRSA prevalence by CF centres and national mean prevalence

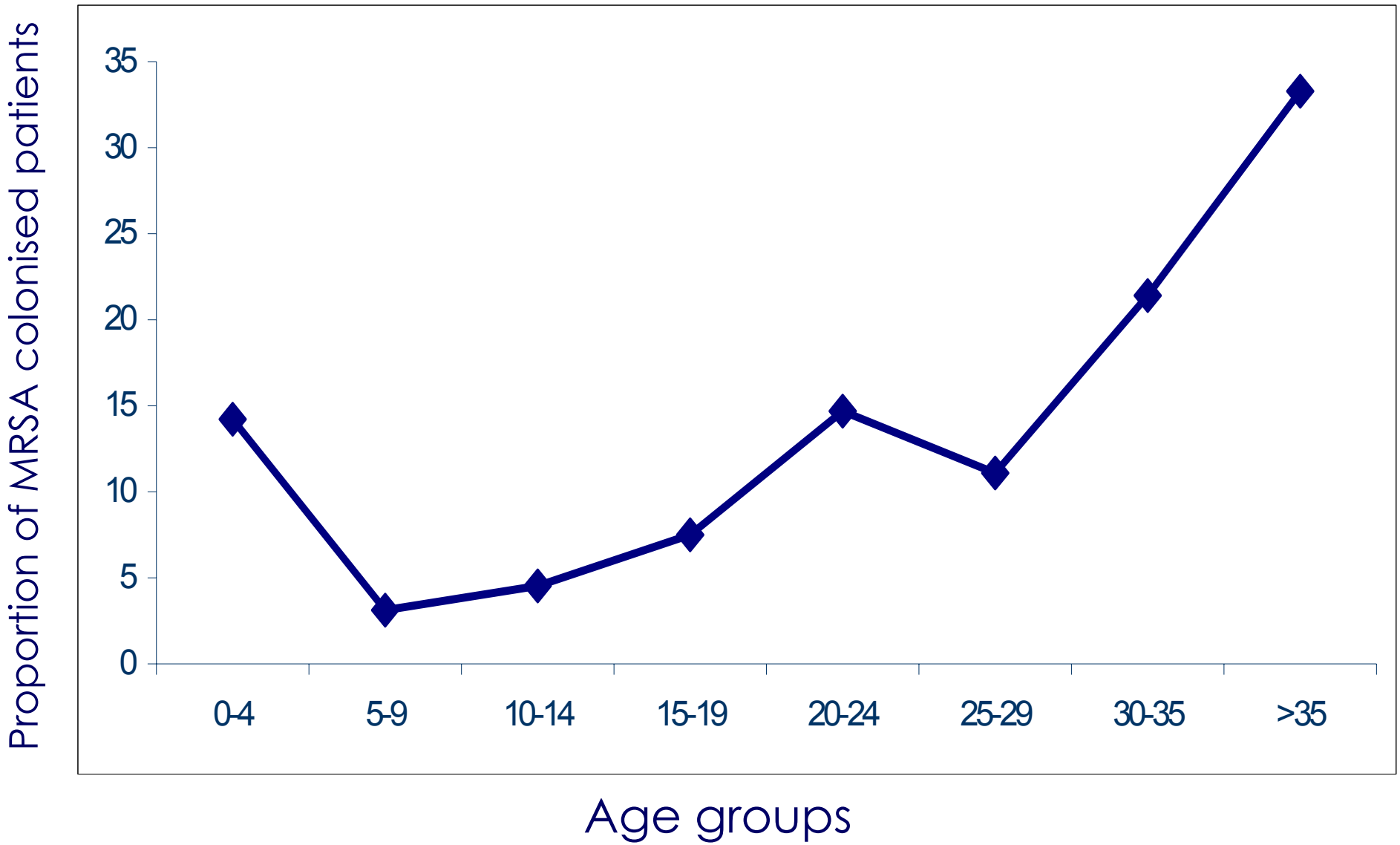


Prevalence of *S. aureus*, MRSA and *S. aureus* SCV colonization by centre

Centre	Mean № of sputum per patient	№ of CF patients with culture	Median age (range in years)	Number of CF patients (%) with		
				S. aureus	MRSA	SCV
1	3.8	20	13 (7-19)	4 (20)	0	0
2	3.4	116	18 (4-40)	33 (28)	6 (5)	0
3	3.6	37	16 (5-39)	11 (30)	0	0
4	5.7	82	15 (5-35)	34 (42)	2 (2)	7 (10)
5	3.1	131	13 (8-27)	23 + 33*(43)	2 (NA)	0 (NA)
6	3.7	81	20 (6-49)	41 (51)	5 (6)	2 (3)
7	2.2	91	15 (0-34)	48 (53)	3 (3)	4 (4)
8	4.3	16	07 (2-11)	10 (63)	0	0
9	5.2	53	22 (10-40)	38 (72)	9 (17)	12 (23)
National	3.75	594 + 33*	17 (0-49)	242 + 33* (44)	27 (5)	25 (4)

*NA = not available; 33 patients whose strains were not available for study

Proportion of MRSA colonised patients in the different age groups



Molecular typing of MRSA

- 20/27 (74%) patients had strains belonging to 4 major epidemic MRSA groups found in Belgian hospitals
  - 12 patients (median age 30 yrs) Group A
  - 4 patients (median age 10.5 yrs) Group B

  (p=0.001 for age comparison)
- 3 « couples » of patients with identical PFGE profiles (2 from same hospitals)

Molecular diversity in patients with MRSA and MSSA

Patient N°	PFGE type	*mec*A	Phenotype	Clonal diversity	Putative event	Known MRSA before the study
1	E2	-	N	Unrelated clones	Loss of MRSA	
	C3b	+	N			
2	C1b	-	N	Unrelated clones	Acquistion of MRSA?	Yes
	A1e	+	N			
3	A22b	-	SCV	Probably related clones	Loss of *mec*A? or co-colonization	Yes
	A20b	+	N			
	K1a	+	N	Unrelated clone	Co-colonization	
4	A23a	-	N	Unrelated clones	Acquistion of MRSA	
	H	+	SCV			
5	F	+	N	Unrelated clones	Co-colonization	
	I1a	-	N			
6	A19b	+	N	Closely related clones	Loss of *mec*A?	
	A19c	-	N			
7	B2a	-	SCV	Closely related/identical clones	Loss of *mec*A?	Yes
	B2d	+	N			
	B2a	-	N			
	B2d	+	SCV			
8	A22a	-	N	Probably related Closely related Identical clones	Loss of *mec*A? or co-colonization	Yes
	A1d	+	SCV			
	A1c	+	SCV			
	A1c	+	N			
9	M1c	+	SCV	Closely related Unrelated clone Closely related clones	Co-colonization	
	E1	-	N			
	M1a	+	SCV			
	M1b	+	SCV			
10	A25a	+	N	Unrelated clones	Acquistion of MRSA?	Yes
	C1a	-	N			
11	A1b	+	N	Unrelated clones	Acquistion of MRSA	
	I1a	-	N			

Distribution of MIC values of 259 *S. aureus* isolates from 242 CF patients

Antibiotic	MIC value mg/l											
	0.06	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
Oxacillin	44	49	60	43	18	10	3	4	3	3	4	18
Vancomycin	16	70	108	53	12	0	0	0	0	0	0	0
Teicoplanin	189	38	15	10	1	6	0	0	0	0	0	0
Gentamicin	97	53	45	29	10	0	1	0	0	0	5	19
Amikacin	0	2	3	8	34	78	60	36	11	15	4	8
Ciprofloxacin	47	30	27	20	27	22	15	22	12	15	9	13
Cotrimoxazole	133	57	26	8	5	1	2	2	2	1	1	20
Linezolid	14	27	100	94	22	1	0	0	0	0	0	0

Characteristics of SCV *S.aureus* strains n = 25

- 5/25 MRSA
- 17/25 showed auxotrophism for
    - 8 thymidine
    - 4 haemin
    - 5 thymidine & haemin
- 8/25 undefined auxotrophism

S.aureus detection methods and prevalence of *S.aureus*/SCV

Method	Centres								
	A	B	C	D	E	F	G	H	I
Specific media for *S.aureus*	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No
S.aureus prevalence (%)	54	56	76	44	28	43	30	63	20
Search for SCV	+-	No	Yes	+-	No	No	No	No	No
SCV prevalence (%)	4,3	2,5	23	8	0	0	0	0	0

SCV detection & identification

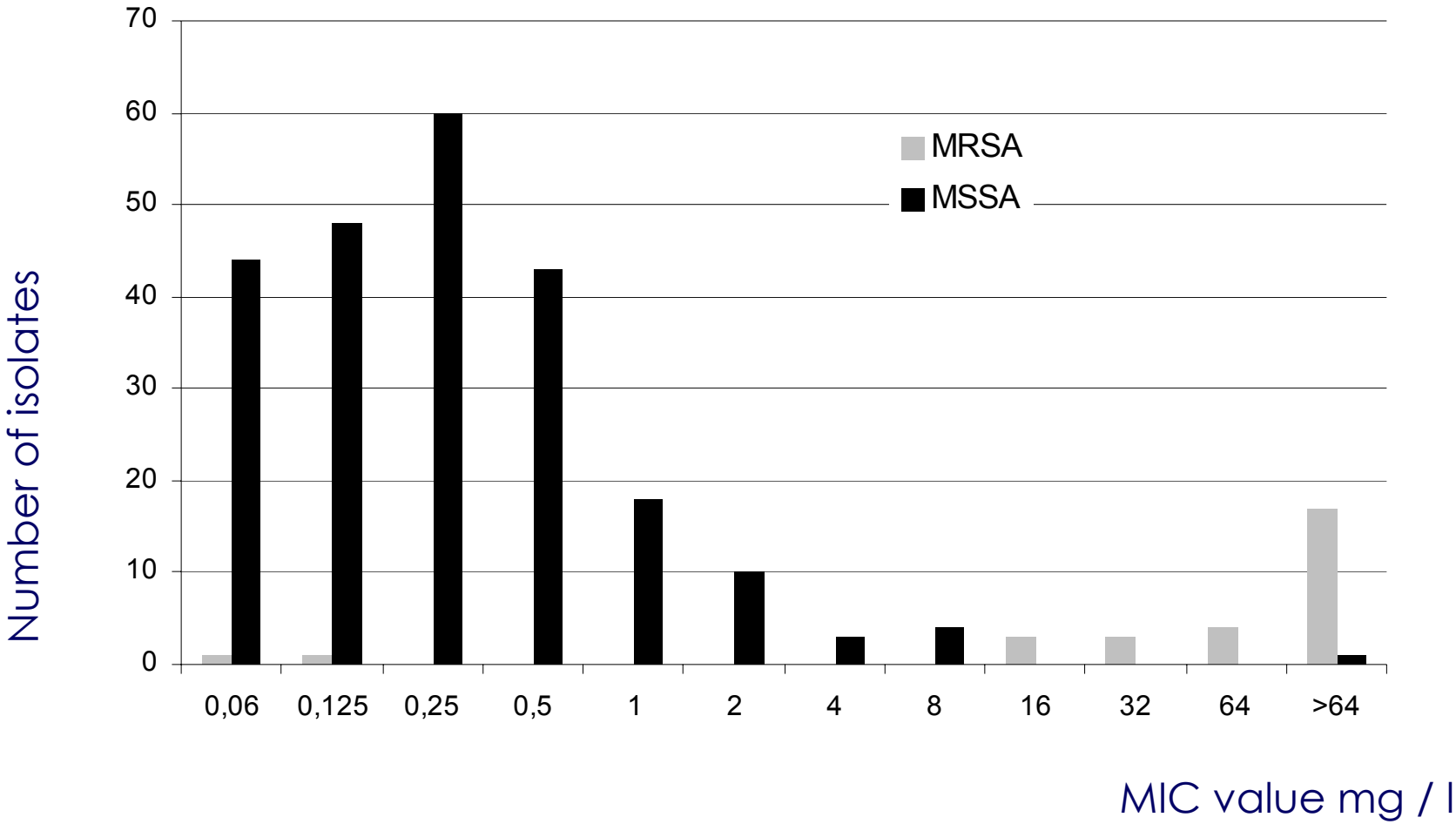
- All grew on MSA (Difco) 24/25 Man +
- 96% coagulase positive (24h)
- 96% agglutination positive
- 100% *nuc* +
- 92% 16S + (vs 100% in nl phenotype)
- 3/5 SCV MRSA did not grow on oxacillin Screen Agar (3/21 nl phenotype = 14%)

Antibiotic susceptibilities of SCV isolates

- 8 mecA- strains had MIC > 4 mg/l
- Apparent R to cotrimoxazole (88%) and S to gentamicin (84%)

  Non-standard medium: false R/S due to restored metabolism?

MIC distribution of MSSA & MRSA



Oxacillin and cefoxitin susceptibility for 9 *mecA* negative strains with oxacillin MIC ≥ 4 mg / l

Strain N°	Oxacillin		Cefoxitin
Normal	Microdilution MH broth+ NaCl	E-test MH+NaCl	E-test MH
1	8	8	8
ATCC29213	0.125	0.25	4
ATCC43300	>64	>256	>256

Strain N°	Oxacillin				Cefoxitin	
SCV	Microdilution MH broth +Vit+NaCl	E-test MH+Vit+NaCl	E-test MH blood	E-test MH blood +NaCl	E-test MH+vit	E-test MH+vit+NaCl
P1A	>64	>256	>256	NG	6	6
P1B	>64	256	1	NG	8	8
P2	4	0.75	2	2	6	4
P3	8	6	1.5	64	8	6
P4	4	6	3	8	2	2
P5	8	8	3	6	8	6
P6	8	NG	2	3	NG	NG
P7	4	4	3	4	4	6
ATCC29213	0.25	0.25	0.38	0.38	4	4
ATCC43300	>64	128	8	128	128	128

Conclusions (1)

- High prevalence of *S.aureus* colonisation (average 43%), but varied widely by centre
- Low prevalence of MRSA colonisation (4.5%) but varied widely by centre (0 to 17%)
- Older CF patients were more likely to be colonised with MRSA
- Most CF MRSA strains are genotypically related to epidemic clones from Belgian hospitals
- 4 MRSA carriers also carried clonally related MSSA (mobile SCC*mec*)

Conclusions (2)

- High ciprofloxacin resistance (33%)
- Prevalence of SCV phenotype (4.2%) lower than in previous studies (but ranging from 0 in 5 centres to 23% in one centre)
- 20% of SCV were MRSA
- 28% of *mecA* negative SCVs had oxacillin MIC ≥ 4 mg/l

Resistance mechanisms? Clinical significance?

Detection of Oxacillin resistance in CF *S. aureus*

- *mecA* PCR = gold standard for MRSA incl SCV
- Latex agglutination = alternative incl for SCV

  (↑ innoculum)
- E-test Oxa or cefoxitin on enriched media in CF
- « BORSA » or « MODSA »
  - Resistance should be characterised for optimal detection
  - Clinical impact should be evaluated

Study limitations and perspectives

- MRSA & MSSA prevalence in CF patients could be underestimated due to use of sub-optimal laboratory methods
- SCV are not detected in many laboratories; further studies are necessary to document their incidence and clinical impact in CF patients
- There is a need for improvement and harmonisation of microbiological procedures in CF patients

Aknowledgments

Participating CF centres (clinician & microbiologist)

AZVUB – Brussels: A. Malfroot & D. Piérard

Huderf – Brussels: G. Casimir & N. Douat

KUL – Leuven: K. De Boeck & J. Van Eldere

St Joseph – Liège: F. Lebrun & F. Pauquay

UCL-St Luc – Brussels: P. Lebecque & J. Gigi

ULB-Erasme – Brussels: C. Knoop & M.J. Struelens

UZ – Antwerpen: K. Desager & M. Ieven

UZ – Ghent: F. De Baets & G. Claeys

Zeepreventorium – De Haan: H. Franckx & T. Calliuw

Investigators

A.Vergison, Pediatric Infectious Diseases, Huderf, Brussels

O. Denis & M.J. Struelens, National Staphylococci Reference Laboratory, ULB- Erasme, Brussels