Problématique des infections à *P. aeruginosa* dans un centre de brûlés

Deuxième partie

L'apport de la biologie moléculaire

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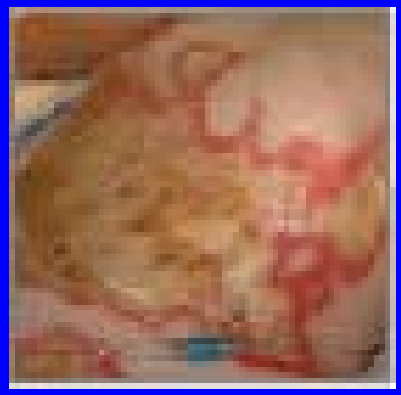
L’apport de la biologie moléculaire

- Diagnostique rapide et plus spécifique
- Meilleur caractérisation des germes en cause
- Outil essentiel pour l’épidémiologie (locale et globale) et la lutte contre les germes R-AB
- Instrument important pour l’hygiène hospitalière

Pseudomonas aeruginosa



Infected Burn Wound

Infected lettuce leaf

- Large genome (6.3 million bp)
- Adapt and colonize
  - Water
  - Soil
  - Rhizosphere
  - Animals
    - Cystic fibrosis
    - Burn
    - Cancer
    - Ventilated ICU patients

History

- 5 August 1998: outbreak MDR O12 *P. aeruginosa*
- Exogenous source suspected
  - → Screening of patients
  - → Environmental survey
  - → Retrospective analysis frozen stock
- 1999: MDR O12 disappears (occupancy rate 10%)

Setting

- ICU: 8 beds, MCU: 24 beds
- Hydrotherapy (5 bathrooms)
- Standard infection control measures
- Sampling: every second day (ICU) or weekly (MCU)
- July 1998 – July 1999: 441 patients (ICU + MCU)

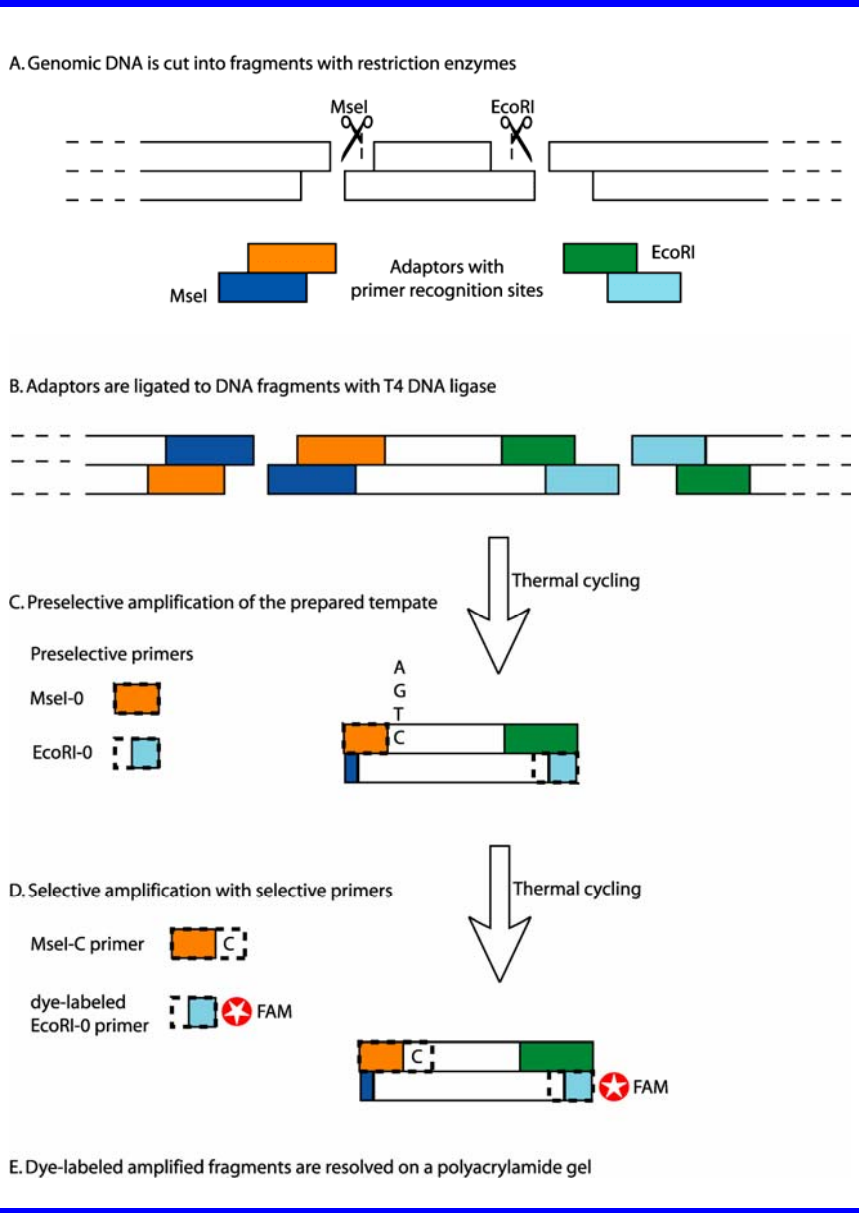
Treatment

- Daily application of silver sulphadiazine (SSD)
- Daily hydrotherapy (sterile water with chlorhexidine)
- Surgical excision 1 to 2 weeks after admission
- First-line (empirical) antibiotic treatment of *P. aeruginosa*
  - aztreonam (ATM) + piperacillin (PIP)
  - ceftazidime (CAZ) + amikacin (AMK)
  - imipenem (IPM) + amikacin (AMK)

Methods

- Standard microbiology procedures
- Serotyping
- Drugsusceptibility testing
  - 11 antibiotics and 5 topicals
- Genotyping:
  - Random Amplification of Polymorphic DNA by PCR (RAPD-PCR)
  - Amplified Fragment Length Polymorphism (AFLP)

AFLP

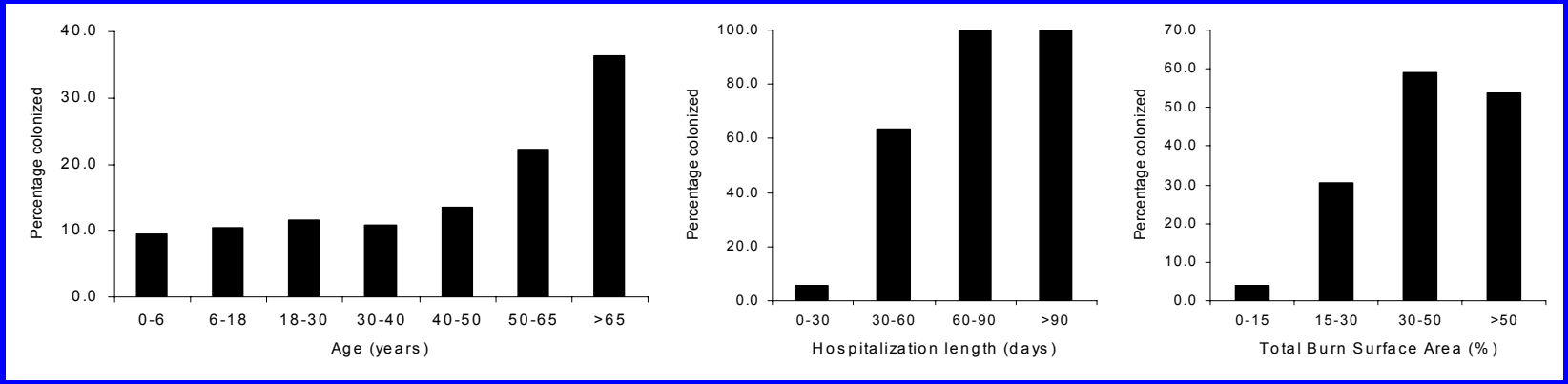

A. Genomic DNA is cut into fragments with restriction enzymes
MseI
EcoRI
MseI
Adaptors with
primer recognition sites
EcoRI
B. Adaptors are ligated to DNA fragments with T4 DNA ligase
Thermal cycling
C. Preselective amplification of the prepared tempate
Preselective primers
MseI-0
EcoRI-0
A
G
T
C
Thermal cycling
D. Selective amplification with selective primers
MseI-C primer
C
dye-labeled
EcoRI-0 primer
FAM
C
FAM
E. Dye-labeled amplified fragments are resolved on a polyacrylamide gel

Results (1)

- Microbiological analysis

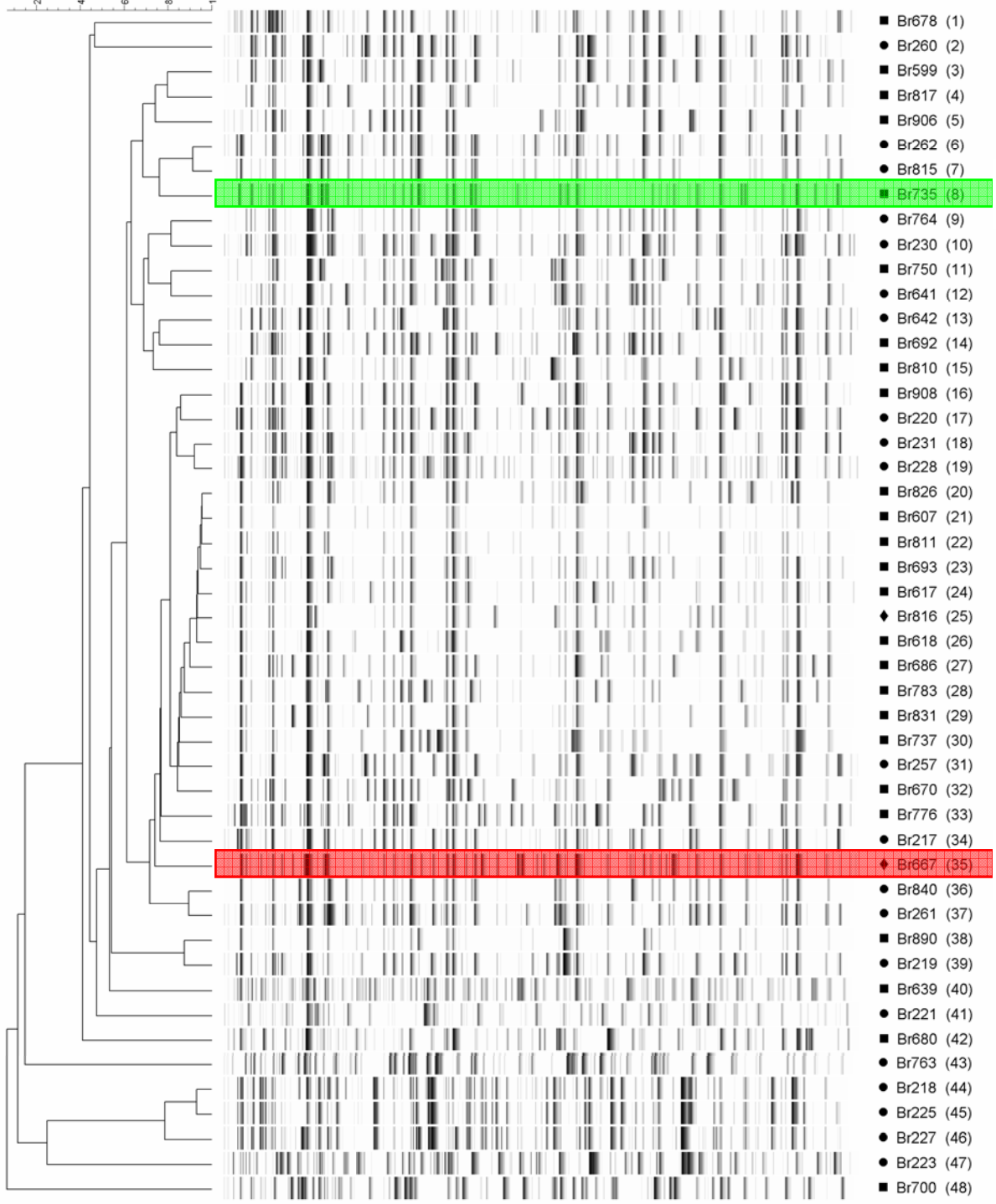
  366 *P. aeruginosa* isolates selected (incl. 45 environmental)

- Characteristics of *P. aeruginosa* colonized patients
  - 70 patients (16%) colonized, 58 (83%) nosocomially
  - Death of 3 patients directly caused by *P. aeruginosa*
  - Colonization ~ age, hospitalization length, and TBSA



Results (2)

- Genotyping
  - 48 AFLP genotypes
    - 15 from individual patients
    - 10 from 2 or more patients
    - 21 exclusively from environment
    - 2 from patients and environment
  - No ongoing *P. aeruginosa* reservoir in environment
  - 57 events of cross-acquisition
  - 19 patients simultaneously colonized by 2 to 4 strains



AFLP8

AFLP35

Normalised AFLP patterns and dendrogram of the 48 genotypes

Results (3)

AFLP35

Epidemic

131 isolates/29 patients

In environment*

MDR

SSD^s

Serotype O12

↔

AFLP8

Epidemic

76 isolates/19 patients

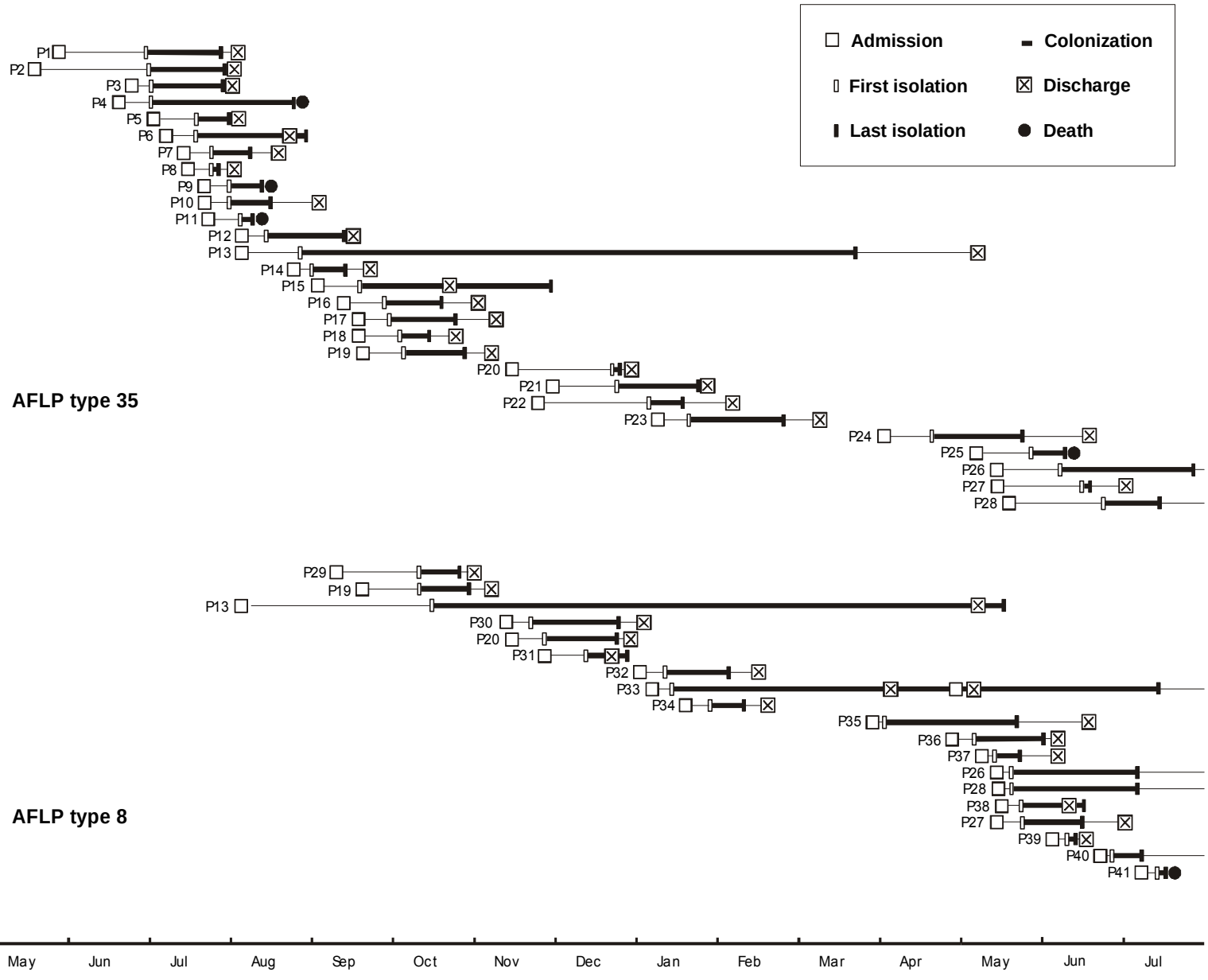
Not in environment

MDS

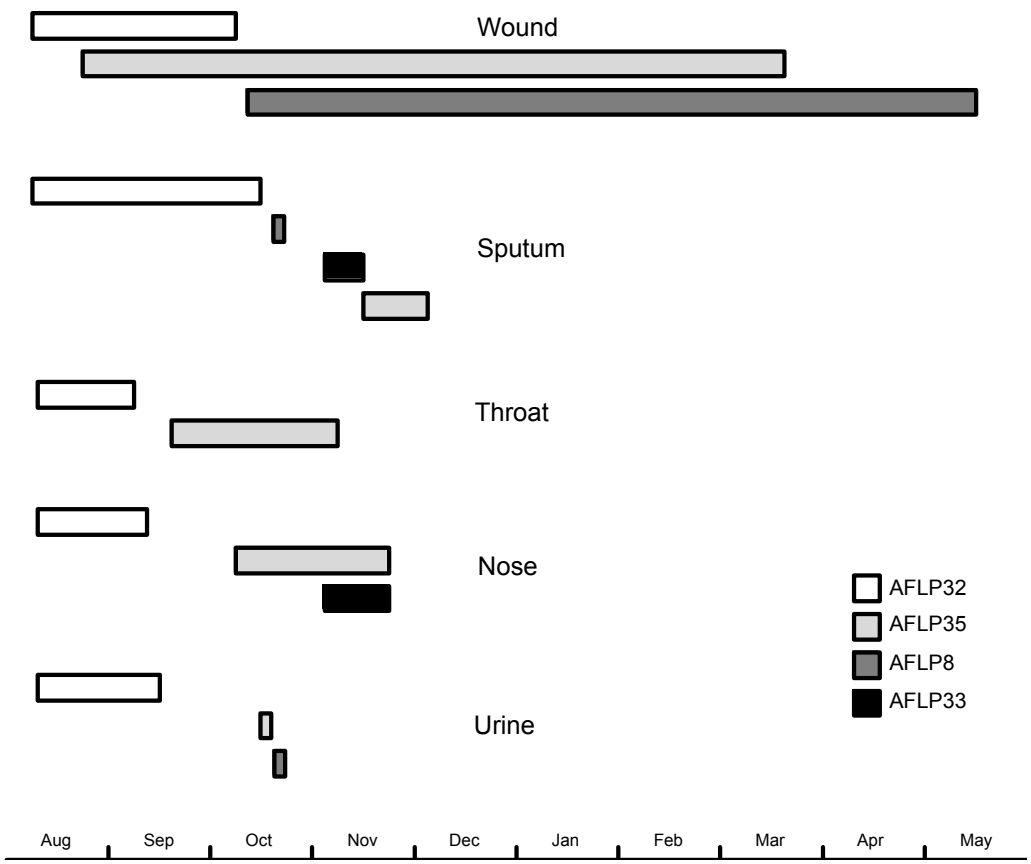
SSD^r

Serotype E

Time course of AFLP35 and AFLP8 colonization



Patient P13 (age 25, TBSA 75%)



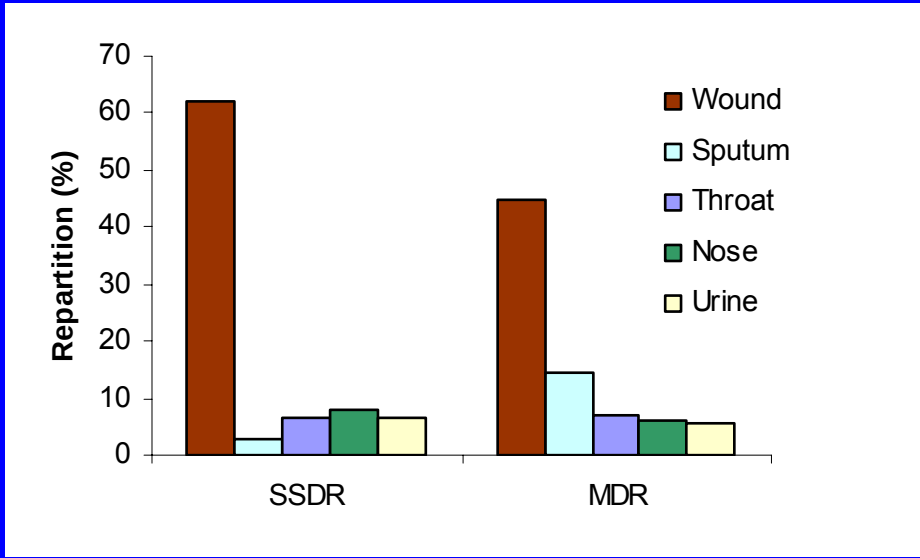
Simultaneously colonised with 4 genotypes (incl. AFLP35 and AFLP8) during his 10 month hospitalization.

Results (4)

- Drug susceptibility testing
  - Antibiotics
    - Most strains susceptible to most antibiotics
    - 4 genotypes (incl. AFLP35) were MDR
    - Acquired resistance to first-line antibiotics
  - Topical agents
    - 8.5% mafenide acetate best *in vitro* activity
    - AFLP8: antibiotic sensitive but SSD^r

Disease habitat selection

Repartition of 126 MDR AFLP35 isolates and 76 SSD^r AFLP8 isolates over different isolation sites



AFLP35 MDR/SSD^s

14% in sputum

45% in burn wounds

AFLP8 MDS/SSD^r

3% in sputum

62% in burn wounds

Discussion

- No inanimate reservoir
- 42 patients (60%) colonized with AFLP35/AFLP8
- Patient P13: continuous reservoir of AFLP35/AFLP8
- Other patients colonized via cross-acquisition
  - e.g. increasing ABR in environmental AFLP35 isolates
- Breaks in barriere nursing techniques
  - shortage of personnel, outdated architecture
- Frequent polyclonal *P. aeruginosa* infections
- AFLP: on 3 July ($\leftrightarrow$ 5 August) 4 patients with AFLP35
- Public health authorities should support genotyping

OprD and carbapenem^{r}

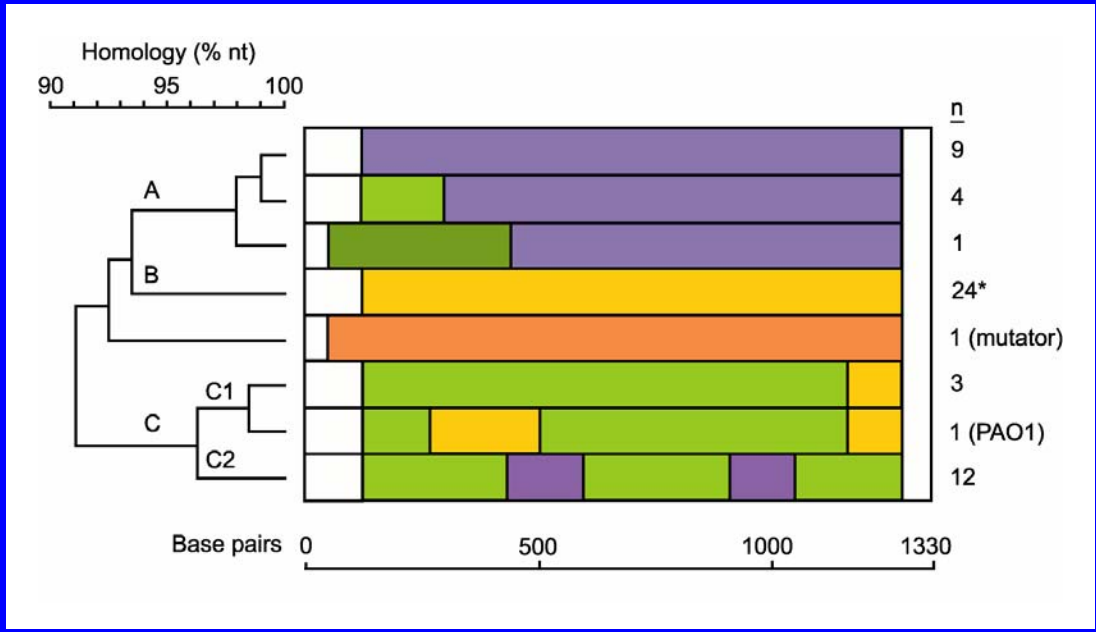
- OprD porin: permeation basic AA and carbapenems
- Carbapenem^{r}: mutational inactivation *oprD*
- 1993 Yoneyama and Nakae: 23 TNPO38 mutants
  - mutation in chromosomal oprD
  - + plasmid with PAO1 *oprD*

  $\rightarrow$ 2 possible mutations (deletions)
- What about natural isolates?

  $\rightarrow$ *oprD* sequence 37 IMP^{s} and 30 IMP^{r}

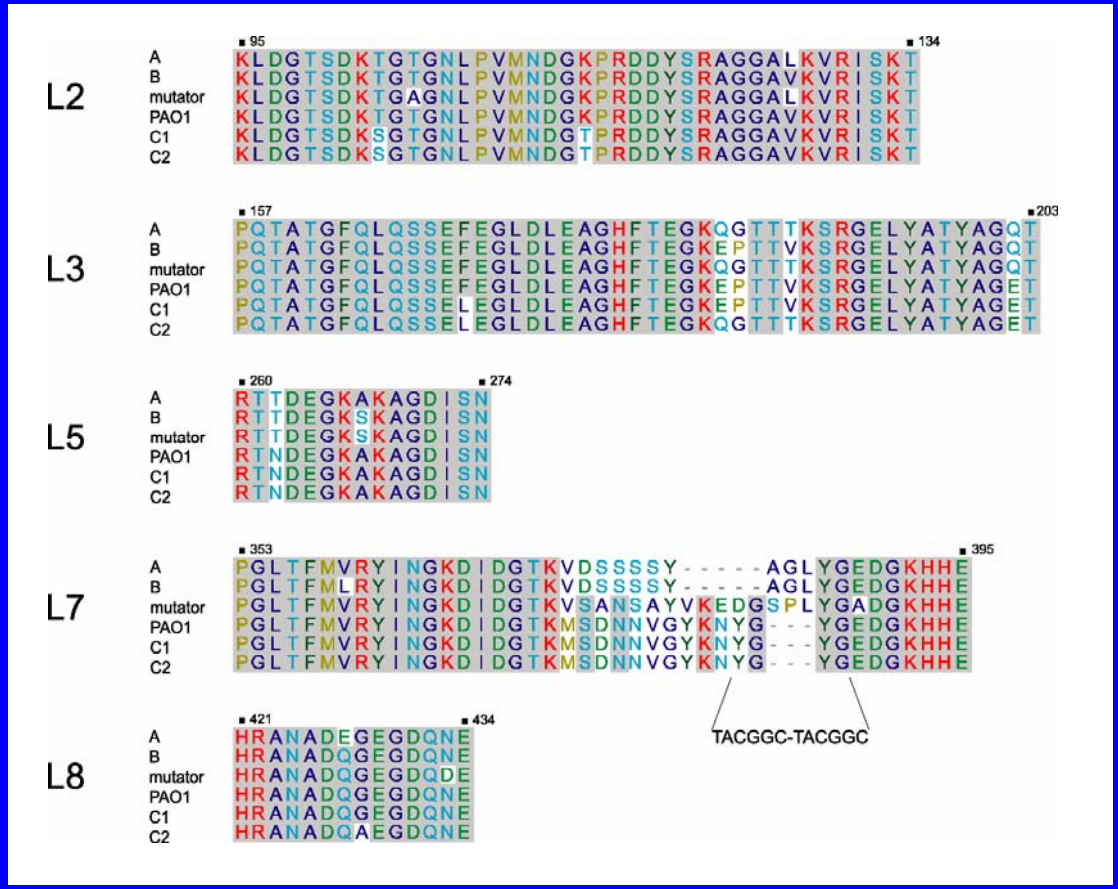
  Spatially separated, clin. and env.

Mosaic structure



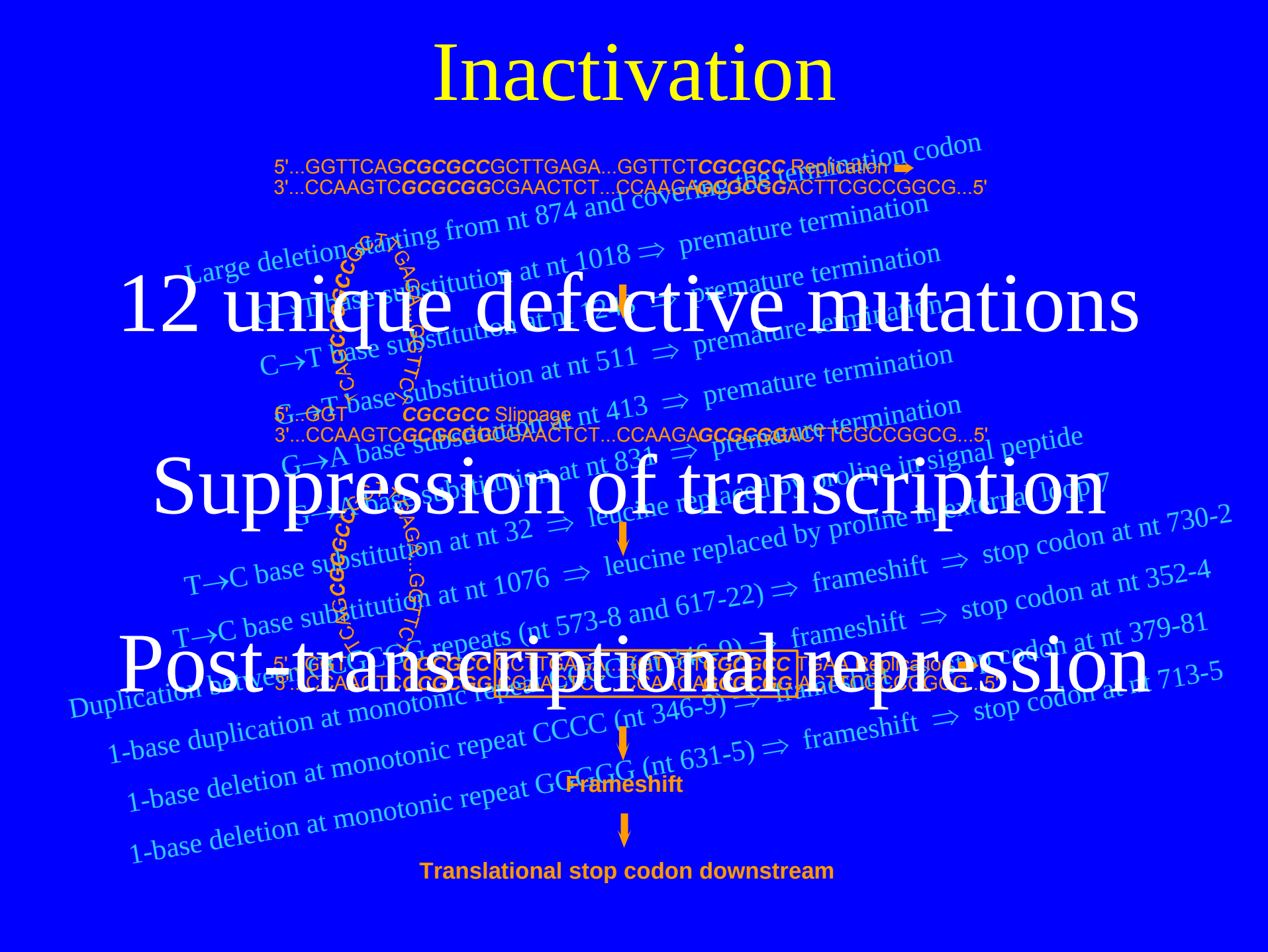
- Mosaic structure (100-300 bp DNA blocks)
- PAO1: distinct sequence, 1.4% from group C
- No correlation group/habitat/geographical origin
- Interspecies exchange probable

External loops



- AA variation high at external loops
- No correlation groups/carbapenem^{r}

Inactivation



12 unique defective mutations

Suppression of transcription

Post-transcriptional repression

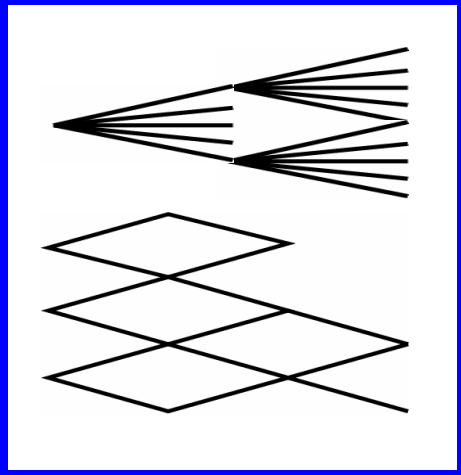
Conclusions

- Important sequence diversity in nature
- Creates complexity
- Careful interpretation of laboratory strain results
- Analyse large batches of natural isolates
- DNA-based assays complicated through complexity

Military Hospital

World

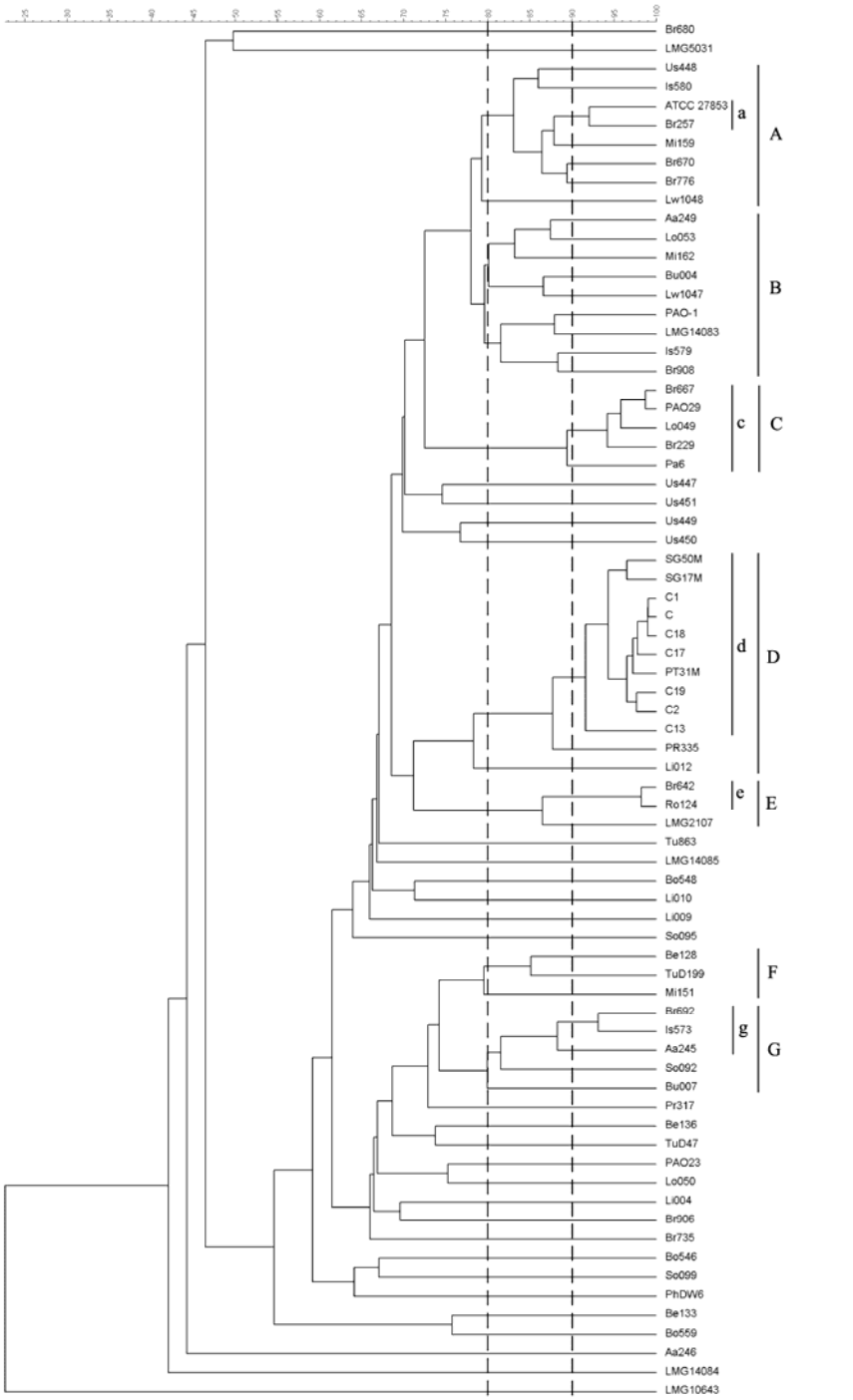
Population structure



Highly clonal

Fully sexual

- Population structure of *P. aeruginosa*?
- 73 clin. and env. isolates from across the world
- Data set:
  - Serotype
  - Pyoverdine type
  - *oprI*/*oprL*/*oprD* gene sequences
  - AFLP fingerprint



Combined analysis

- Biological data analysis software
- Clonal complexes (≥ 80% homol.)
- Unique isolates
- No correlation
  habitat/geographical origin
- Recombinations
- Network of relationships
- Subclusters (clones) ≥ 90% homol.

Conclusions

- Non congruence of experiments
- Network of relationships
- Direct evidence of recombination (*oprD*)
- Clones with nearly identical data set

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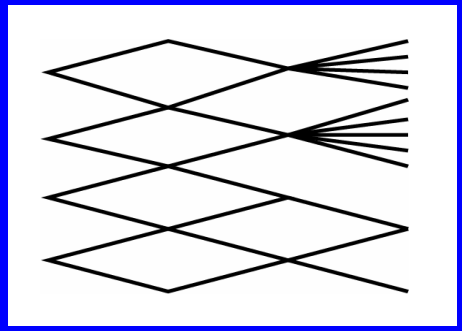
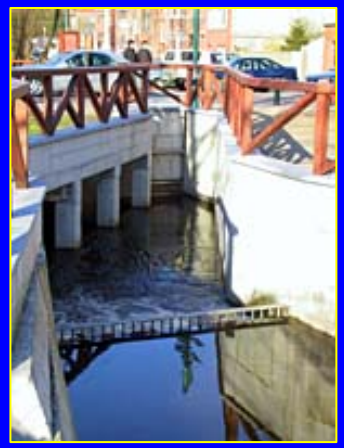


Epidemic population structure

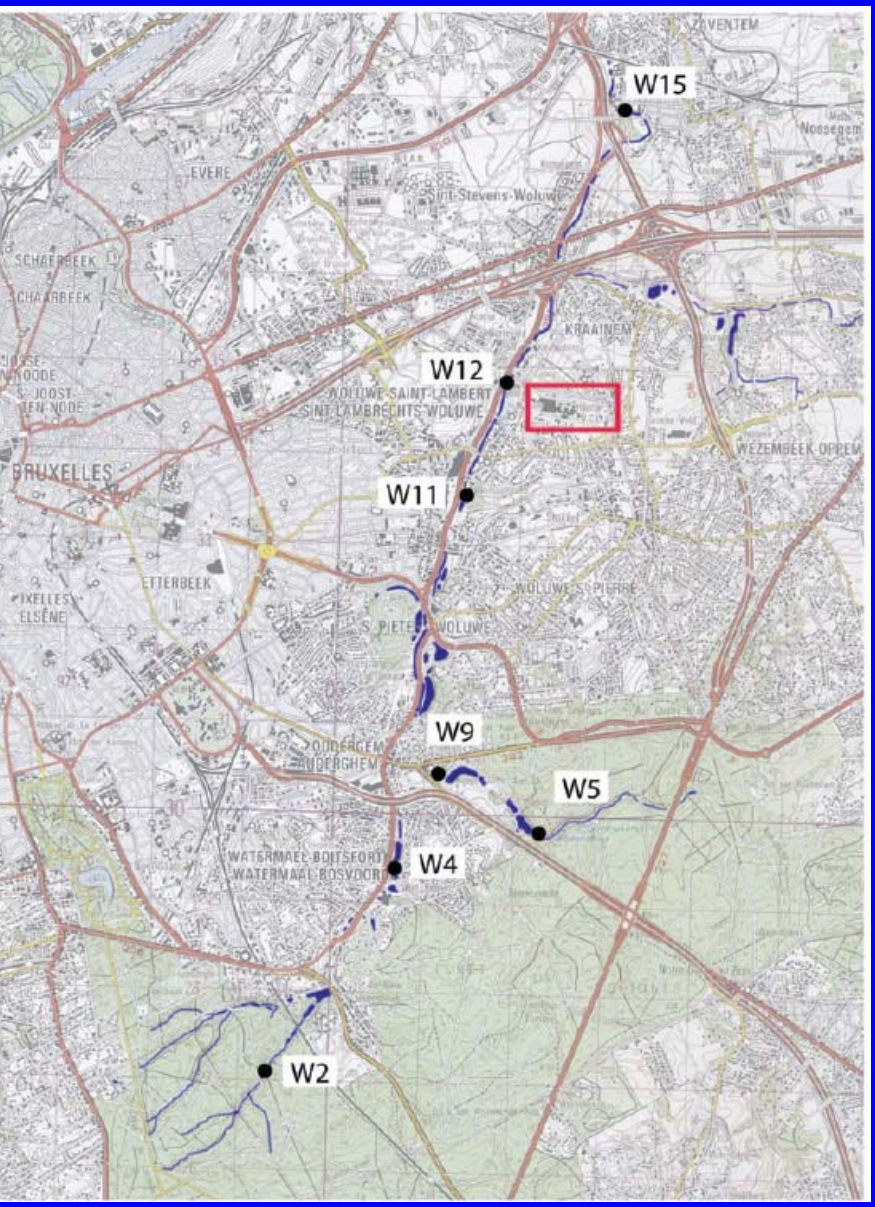
Military Hospital

Environment

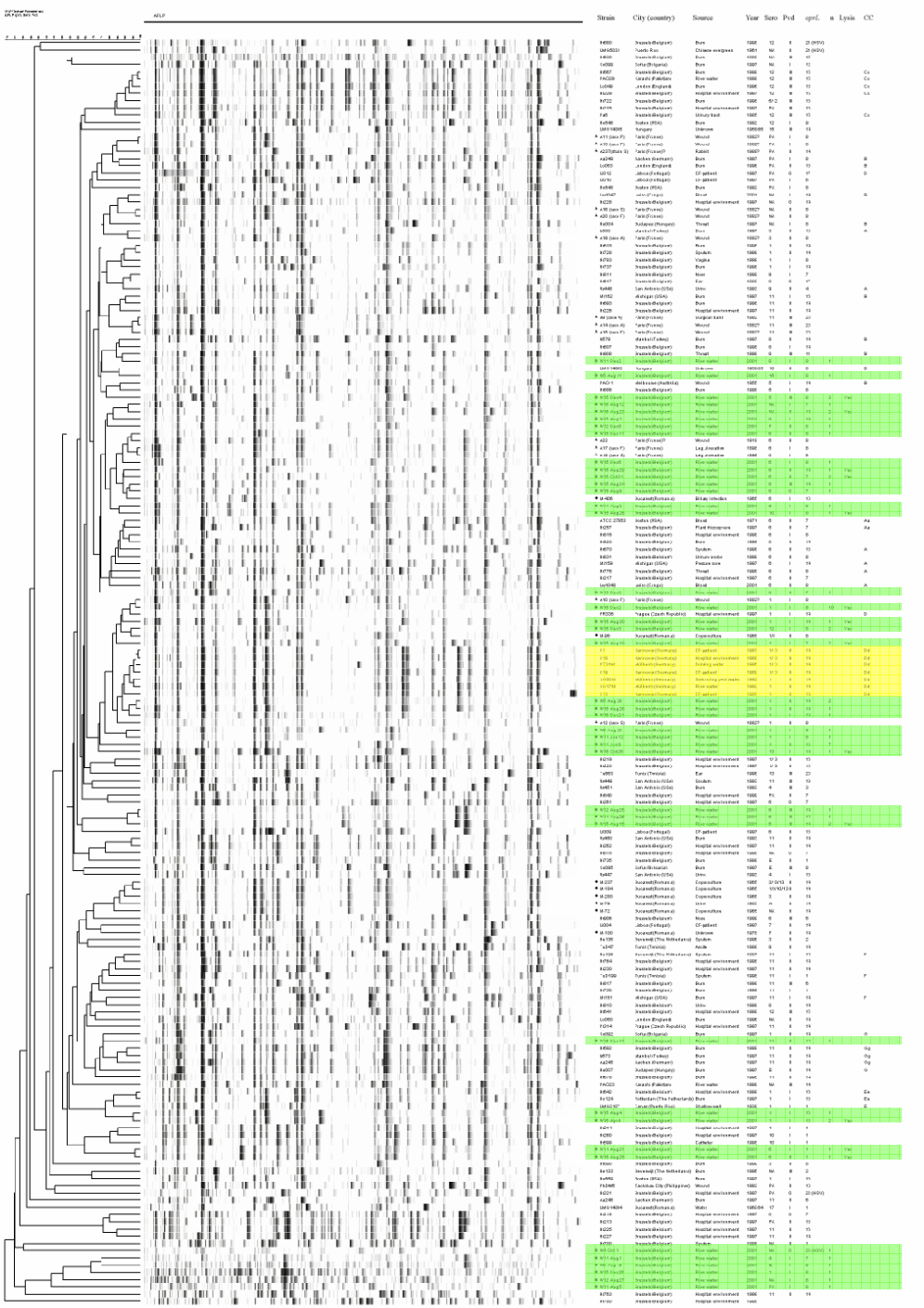


World

Environment: river Woluwe



- Brussels
- Length: 15 km
- 5 Sources in forrest
- Mouth: Zenne river in Brussels
- Downstream pollution: agriculture/roads/domestic/industrial
- Sampling:
  - 7 locations
  - Every 2 months for 1 year



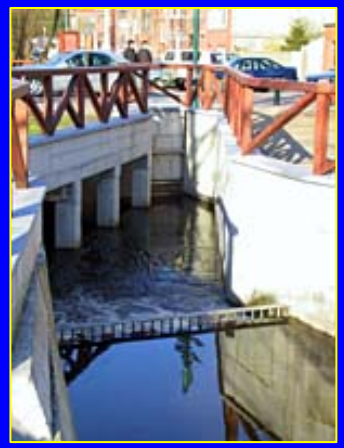
Woluwe

CF/Aquatic Germany

- 41 distinct strains (AFLP/*oprL*/*oprI*/Pvd/Sero)
- ~ Pollution
- Important diversity ‘Woluwe strains’
- Some related to German clone

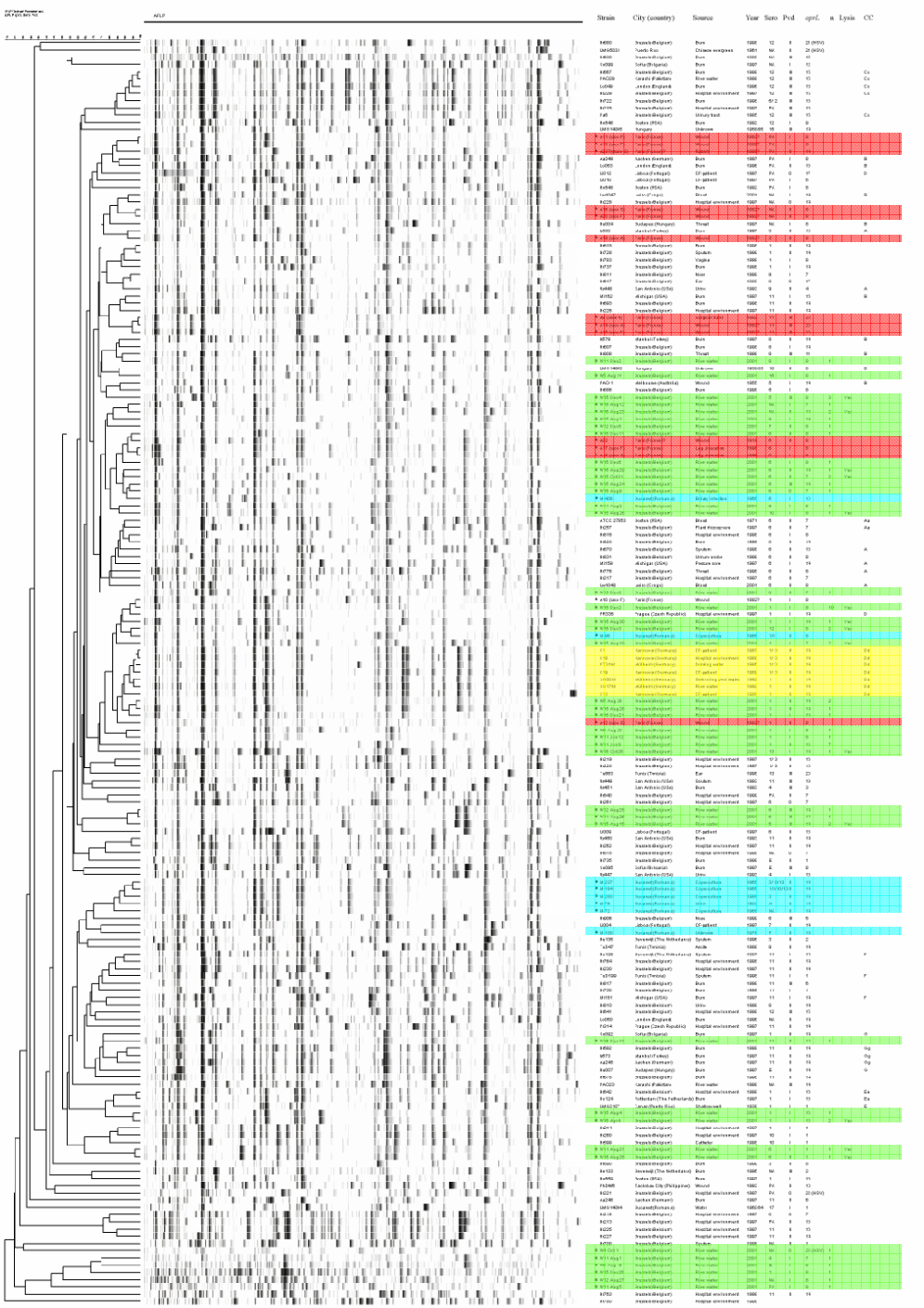
Military Hospital

Environment



World

Tenability



Woluwe

CF/aquatic Germany

Pasteur

Meitert

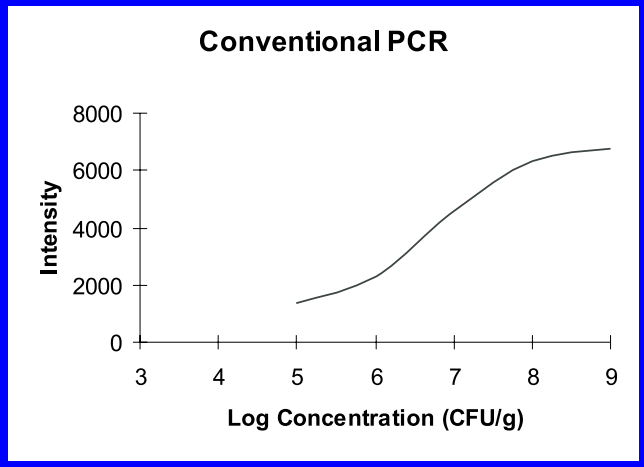
- No clustering Pasteur/Meitert strains
- 19th century *P. aeruginosa* hospital outbreak?

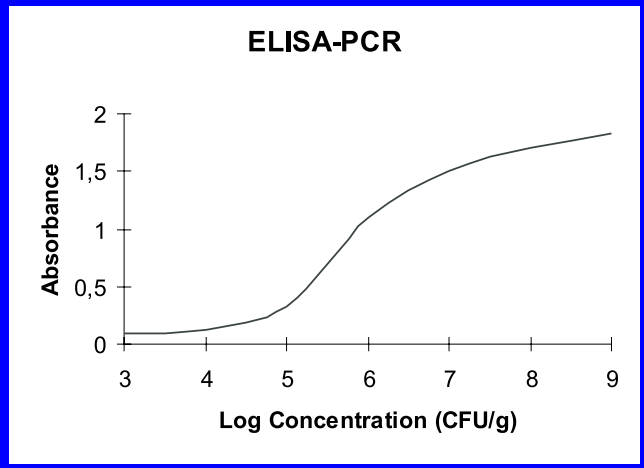
Molecular Diagnostics

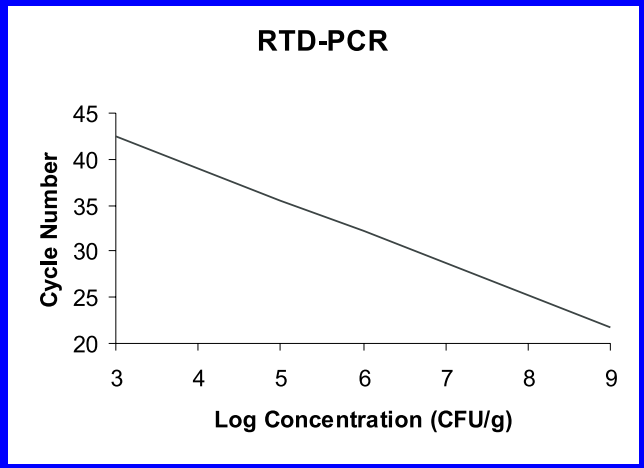
- Diagnosis of Disease using the molecular components (genome…)
- i.c. microbial infection(s): bacterial, fungal, viral
- - Advantages: quicker, detects not / or difficult growing agents
- - Disadvantages: does presence of DNA = presence of disease???
- ex.: Blood Stream Infections (BSI)…

Real-time PCR (1):

Standard curves for reconstituted biopsy samples







Real-time PCR (2):

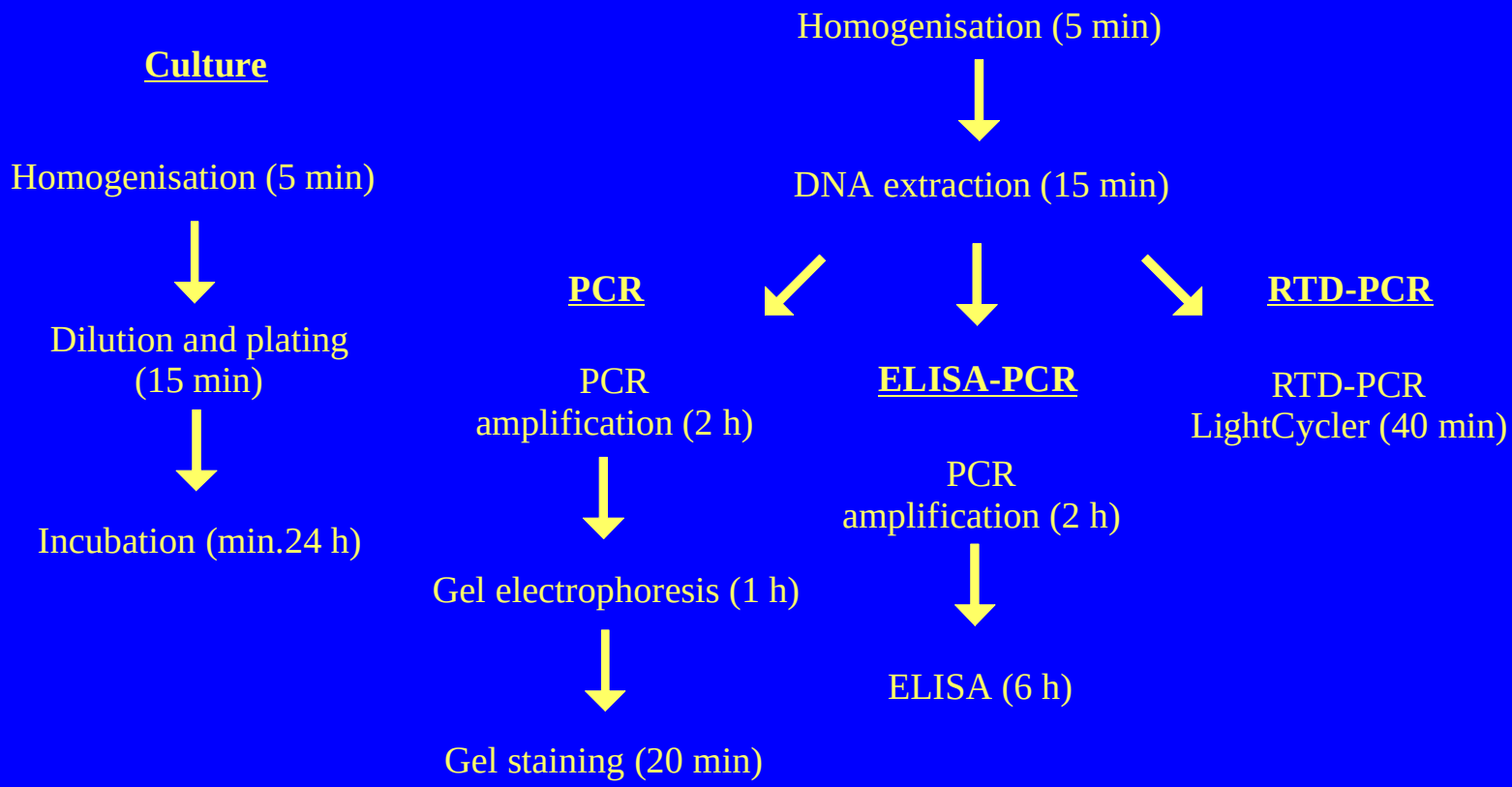
Summary of the characteristics of the tested quantitation methods.

Method	Lower detection limit[1]		Log-linear range (CFU/g)	Time to result (hours)	Ease of use	Cost per test ($) [3]	Set up cost ($)
	(CFU/g)	(CFU/reaction)					
Culture	10^2	1	10^2-10^9	24	Easy	6	3.500
PCR	10^4-10^5	10-10^2	10^6-10^8	4	Moderate	12 [4]	20.000
ELISA-PCR	10^3-10^4[2]	1-10	10^5-10^7	8	Elaborate	25 [4]	20.000
RTD-PCR	10^3-10^4	1-10	10^3-10^9	1[2]	Moderate	9 [4]	73.000

[1] The lower detection limit is variable due to variations in inter-run amplification efficiency.
[2] When using a rapid thermal cycling technique.
[3] Does not include labour costs, only reagents and disposables.
[4] Cost per clinical sample, based on the analysis of batches of 10 clinical samples and 8 standards.

Real-time PCR (3):

Comparison of the procedural steps involved in the quantitation methods



General conclusions

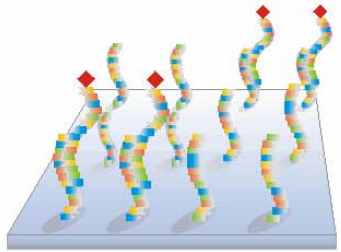
- Routine fingerprinting could have prevented the *P. aeruginosa* outbreaks in the BWC
- Large batches of clin. and env. isolates → more reliable
- *P. aeruginosa* displays an epidemic population structure
- Antibiotics → rapid selection and spread of MDR clones
- Anatomic habitat selection

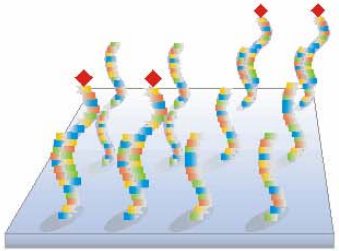
SNP-Chip: Detection

2 loops of bacteria from an Agar- plate, washed and resuspended

Multiplex-PCR and labelling with 100 specific and optimized oligonucleotides

Hybridization against the *P.aeruginosa* AT-Chip





Conjugation and colorimetric detection

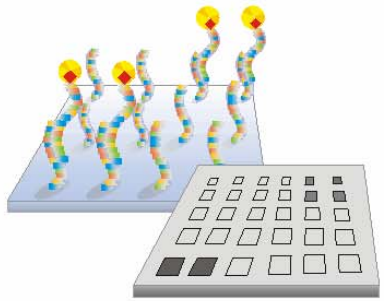
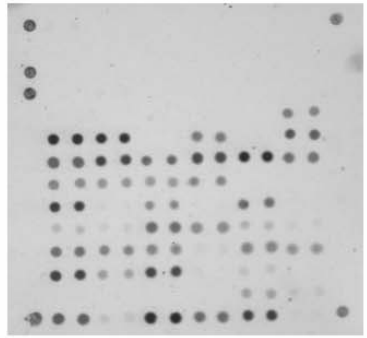
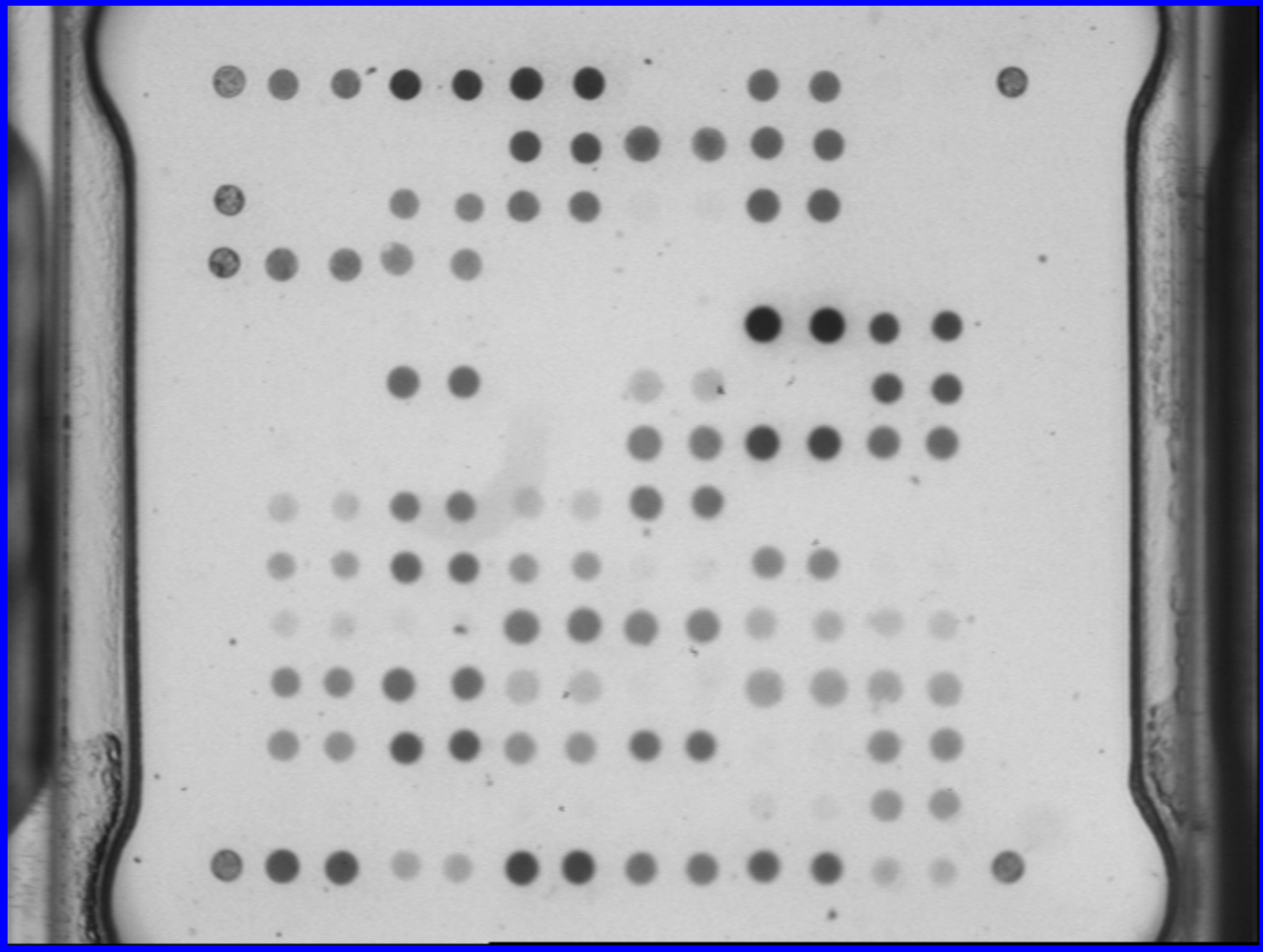


Image acquisition and analysis



SNP-Chip: Results



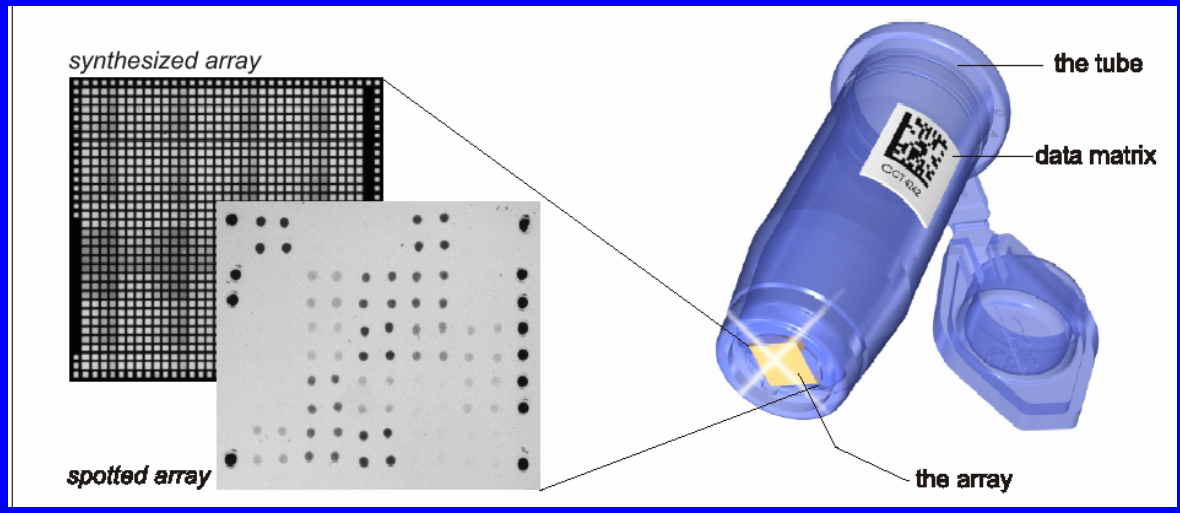
Characteristics:

fliC type b
no fla- islands 1+2
exoS
PAPI-1
no PAPI-2
PAGI-1
PAGI-2/3- type island
C45, C46, C47
but no PAGI-2 or 3
pKLC102- type plasmid

PA3835
no PA2221
PA2185
PA0980
no PA0728
no PA0722
no PA0636

SNP“63741“: 000-XX1-111-100-010-011

Characteristics of Clondiag® AT-array



- A low resolution DNA Chip is embedded in the bottom of an eppendorf-like tube (Clondiag® **A**rray **T**ube).
- All hybridization and washing steps are performed in this tube.
- Results, obtained by silver precipitation or peroxidase reaction, are also read in the tube.
- Stable grey images, given by silver precipitates, allows examination with various transmission imaging readers (scanners, microscopes, etc.) and long- time storage.