MASTERPROEF
'VALUE OF PNEUMOCOCCAL PCR IN DIAGNOSIS OF PARAPNEUMONIC PLEURAL EFFUSION'

15/05/2012

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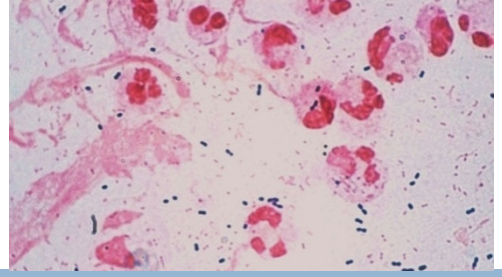
Overview

1. Introduction
2. Study objectives
    1. Evaluation of **real-time PCR** for the detection of *S. pneumoniae* in pleural fluids
    2. **Pneumococcal antigen detection** (Binax NOW®) in pleural fluids
    3. Capsular serotyping of *S. pneumoniae* directly on **cultures**
    4. *Capsular serotyping* of *S. pneumoniae* directly on **pleural fluids**
    5. **Cost- and time-effectiveness**
3. Limitations
4. Conclusion
5. To do's

Introduction



- ***S. pneumoniae*:** major bacterial pathogen causing severe infections with high morbidity and mortality
  - ⇒ 1,2 million deaths/year
- **Community-acquired pneumonia**
  - Postpneumonic empyema in pediatric children: 0,7 - 9%
- **Microbiology:**
  - Culture: golden standard
    - 24-48h to confirm diagnosis
    - High specificity, low sensitivity
  - Gram stain
- **Polymerase chain reaction**
  - Higher sensitivity
  - Unaffected by prior administration of antibiotics
  - Fast

Serotyping

- The immunochemistry of this capsular polysaccharide ⇒ **93 distinct capsular serotypes**

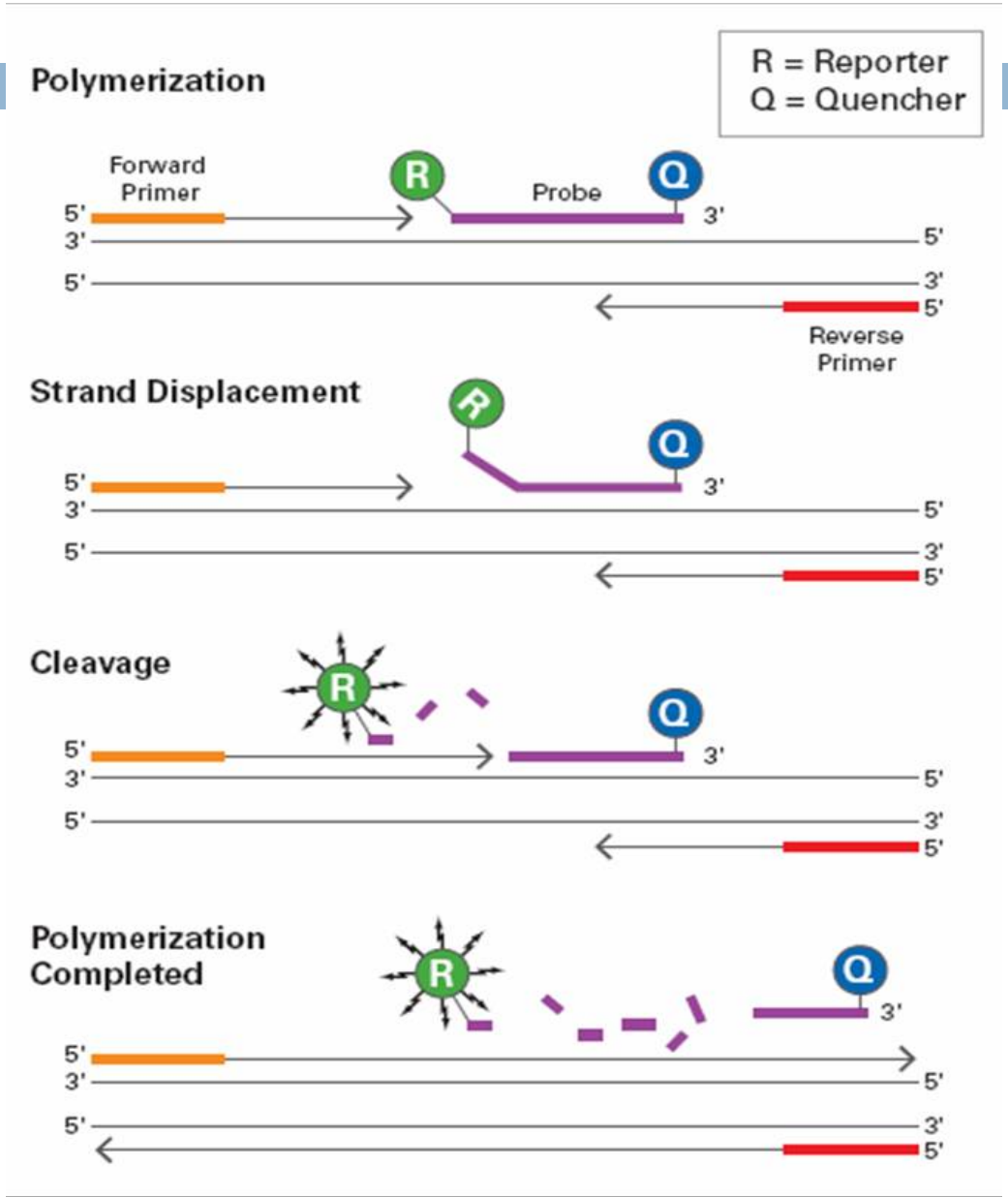
  ⇒ **15 serotypes:** the majority of invasive pneumococcal disease

- Serogroup specific epidemiology ⇒ development of future conjugate vaccines
  - 2007: **7-valent vaccine (PCV 7; Prevenar 7®)** implemented in Belgium
    - 3 doses: 2,4 and 12 months
    - Serotypes 4, 6B, 9V, 14, 18C, 19F and 23F
  - 2010: **13-valent vaccine (PCV 13; Prevenar 13®)**
    - Serotypes PCV 7 and 1, 3, 5, 6A, 7F and 19A

PART 1

Evaluation of *autolysin* real-time PCR for detection of *S. pneumoniae* in pleural fluids

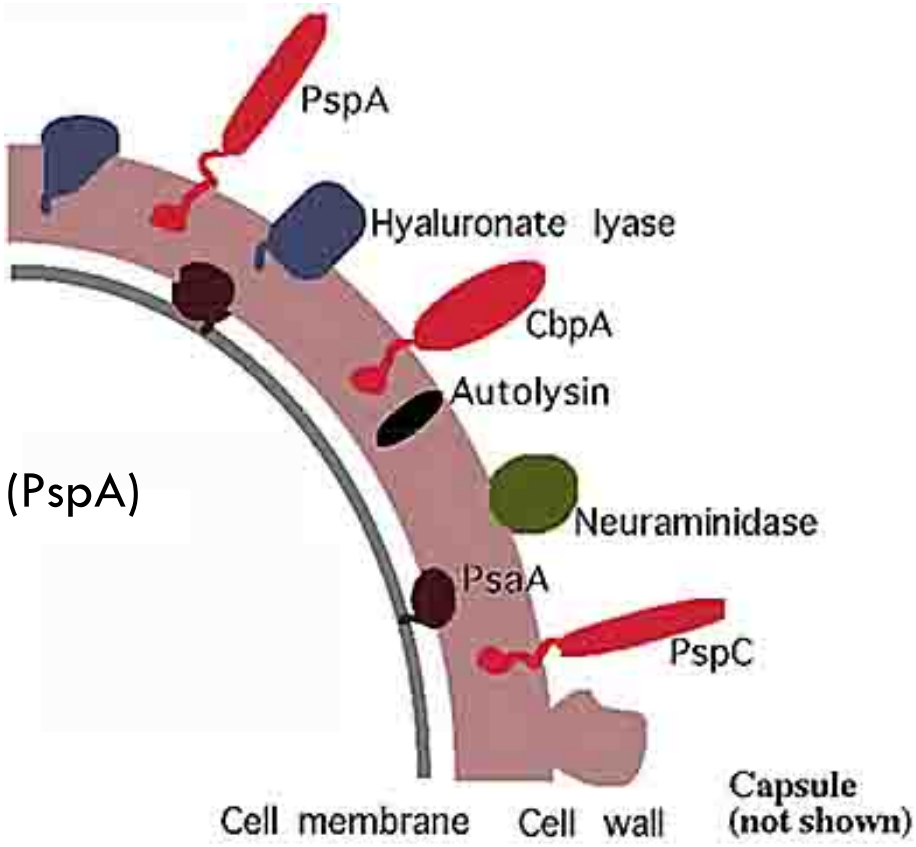
Real-time PCR



Autolysin (lytA) PCR

Virulence factors

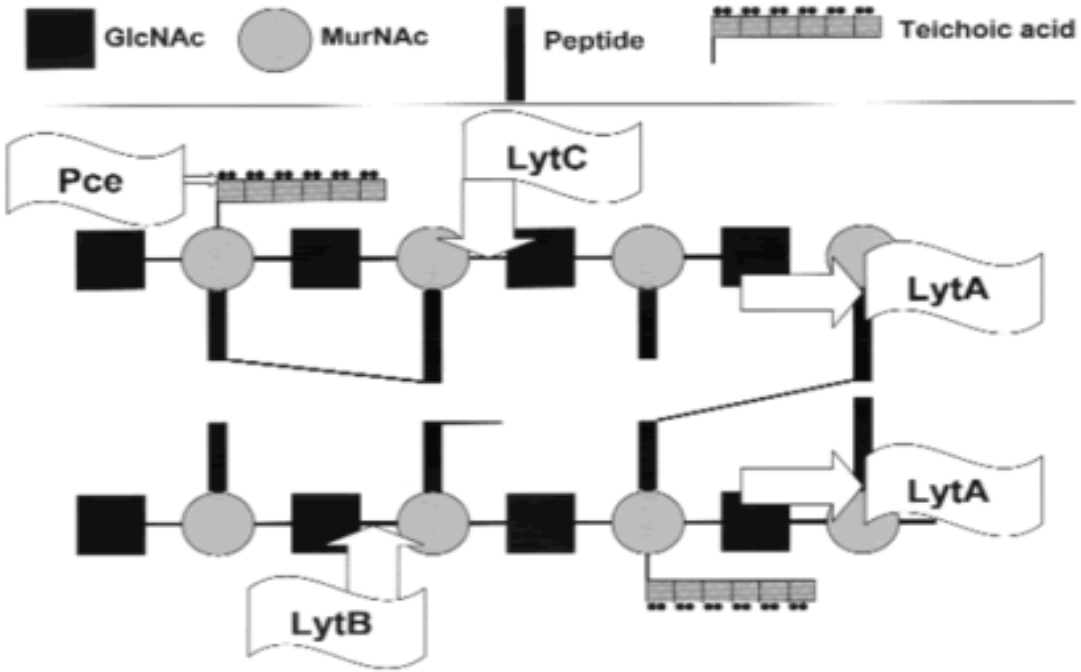
- **Polysaccharide capsule**
- **Pneumococcal proteins:**
  - Pneumolysin (*ply*)
  - Neuraminidase
  - Autolysin (*lyt*A)
  - Surface proteins
    - Pneumococcal surface protein A (PspA)
    - Pneumococcal adhesines
  - Other proteins:
    - Hyaluronidase
    - IgA 1 protease



Autolysin (*lyt*A)

- Powerful autolytic enzyme
- Degradation of different bonds in peptidoglycan

  ⇒ lysis and cell death
- Release of lipoteichoic en techoic acids (mediators of host inflammatory respons)



Implementation of real-time PCR

Specificity of PCR assays

	Encapsulated pneumococci	Nontypeable pneumococci	Atypical *Streptococci*	Closely related viridans and *D. pigrum*
lytA	40/40 (100%)	4/4 (100%)	0/16 (100%)	0/35 (100%)
psaA	40/40 (100%)	4/4 (100%)	1/16 (96%)	0/35 (100%)
IAIB *ply*	40/40 (100%)	4/4 (100%)	8/16 (50%)	0/35 (100%)
IIAIIB *ply*	40/40 (100%)	4/4 (100%)	16/16 (0%)	0/35 (100%)

Messmer et al. *Diagn Microbiol Infect Dis.* 2004 Aug;49(4):249-54

UZ LEUVEN: *Lyt*A PCR study protocol

- **Patients and methods:**
  - Retrospective
  - 31 children and adolescents with parapneumonic pleural effusions (UZ Leuven from 4/2008 to 11/2010)

- **DNA extraction / PCR reaction:**
  - Extraction: Nuclisens easyMAG (Biomérieux)
  - PCR reaction:
    - Primers for the pneumococcal autolysin gene (*lyt*A gene) of *S. pneumoniae*
    - FAM/TAMRA-labeled probe
    - ABI 7500 Sequence Detection System device

Comparison of PCR and culture results for *S. pneumoniae* in samples of pleural fluid (n=31)

	Positive Culture	Negative Culture	Total
Positive *lytA* PCR	0	23	23
Negative *lytA* PCR	1	4	5
Inhibition *lytA* PCR	1	2	3
Total	2	29	31

P. mirabilis

S.pyogenes

Comparison between *lyt*A PCR results and Gram stain

	Gram stain				
	Negative	**PMN cells ++**	**PMN++, GPC++**	**No Gram stain**	**Total**
Positive PCR	8	6	0	9	23
Negative PCR	2	0	0	3	5
Inhibition PCR	1	1	**1**	0	3
Total	11	7	1	12	**31**

S.pyogenes

PART 2

Pneumococcal antigen detection (Binax NOW®) in pleural fluids

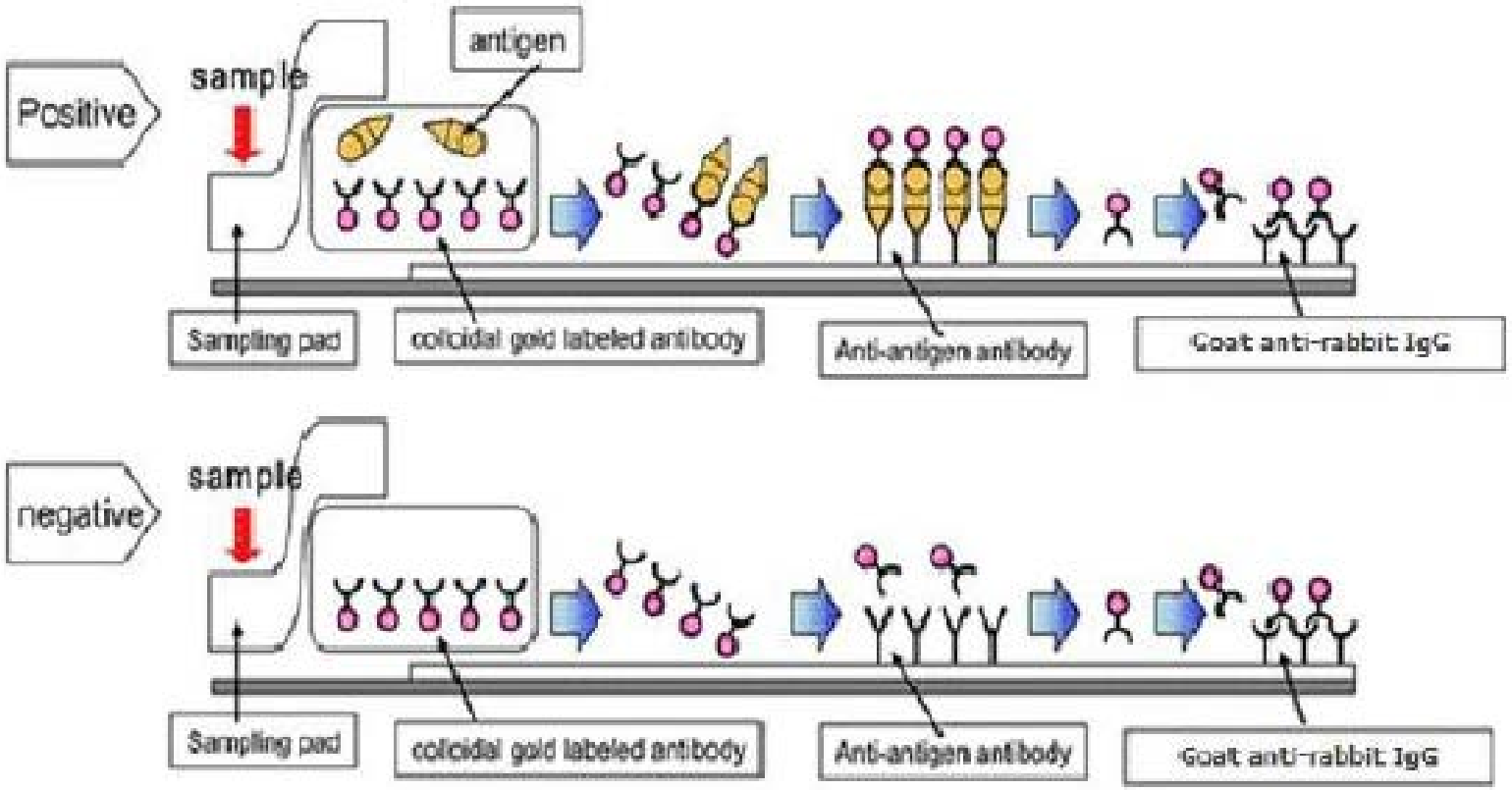
2. BinaxNOW® *Streptococcus pneumoniae*

- BinaxNOW® : **rapid immunochromatographic membrane assay**
  ⇒ detection of C polysaccharide cell wall antigen of *S. pneumoniae*

- BinaxNOW® *S. pneumoniae* has been validated for detection of *S. pneumoniae*:
  - ➢ in the **urine** of patients with pneumonia
  - ➢ in the **cerebrospinal fluid** of patients with meningitis

- **Aim of this study:**
  - Evaluation of Binax NOW test on the same 31 pleural fluids
  - Comparison between Binax NOW test and lytA PCR

BinaxNOW® test principle



BinaxNOW® test result

BinaxNOW® test procedure

- Just before testing mix specimen by gentle swirling
- Dip swab into specimen, touch swab on side of container to remove excess fluid
- Insert swab into bottom hole and push up so swab is fully visible in the top hole.
  DO NOT REMOVE SWAB.
- Holding Reagent A upright slowly add 3 drops to the **bottom hole**
- Immediately peel off brown adhesive liner and close test device
- Read results at **15 minutes**

BinaxNOW® literature

BinaxNOW in pleural fluids						
Author	**Year**	**Country**	**No of samples**	**PCR**	**Sensitivity**	**Specificity**
Le Monnier et al.	2006	France	40	16S rDNA PCR	97,5%	95%
Ploton et al.	2006	France	69	16S rDNA PCR	100%	83,3%
Hernandez-Bou et al.	2009	Spain	59	*ply* RT-PCR	100% (culture) 87,8% (PCR)	100% (culture/PCR)
Flores et al.	2009	Spain	73	*ply* RT-PCR	88%	71%
Strachan et al.	2011	Australia	130	*lyt*A PCR	83,8%	93,5%

BinaxNOW® on 27 pleural fluids: results

		PCR RESULT		
		Positive	Negative	Total
BINAX				
	Positive	21	0	21
	Negative	1	5	6
Total		22	5	27

Sensitivity, 95,5%; Specificity, 100%; PPV, 100%; and NPV, 83,3%

Ct value *lyt*A PCR: 36,4

PART 3

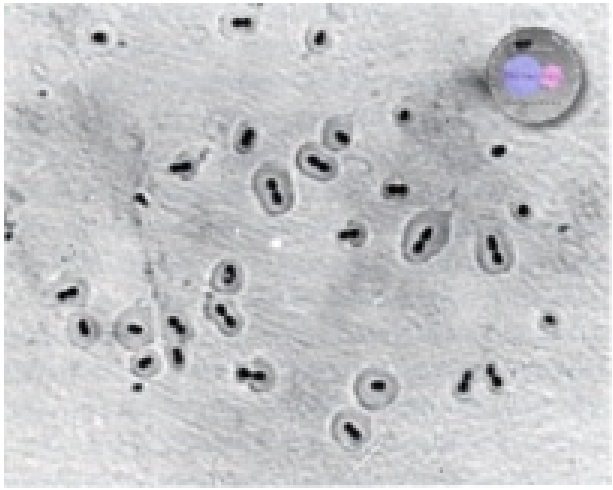
Capsular serotyping of *S. pneumoniae* directly on cultures

Capsular serotyping

- Current practice UZ LEUVEN: **QUELLUNG REACTION**
  - Gold standard for pneumococcal serotyping
  - Cultured organisms
  - Binding of specific monoclonal antibodies

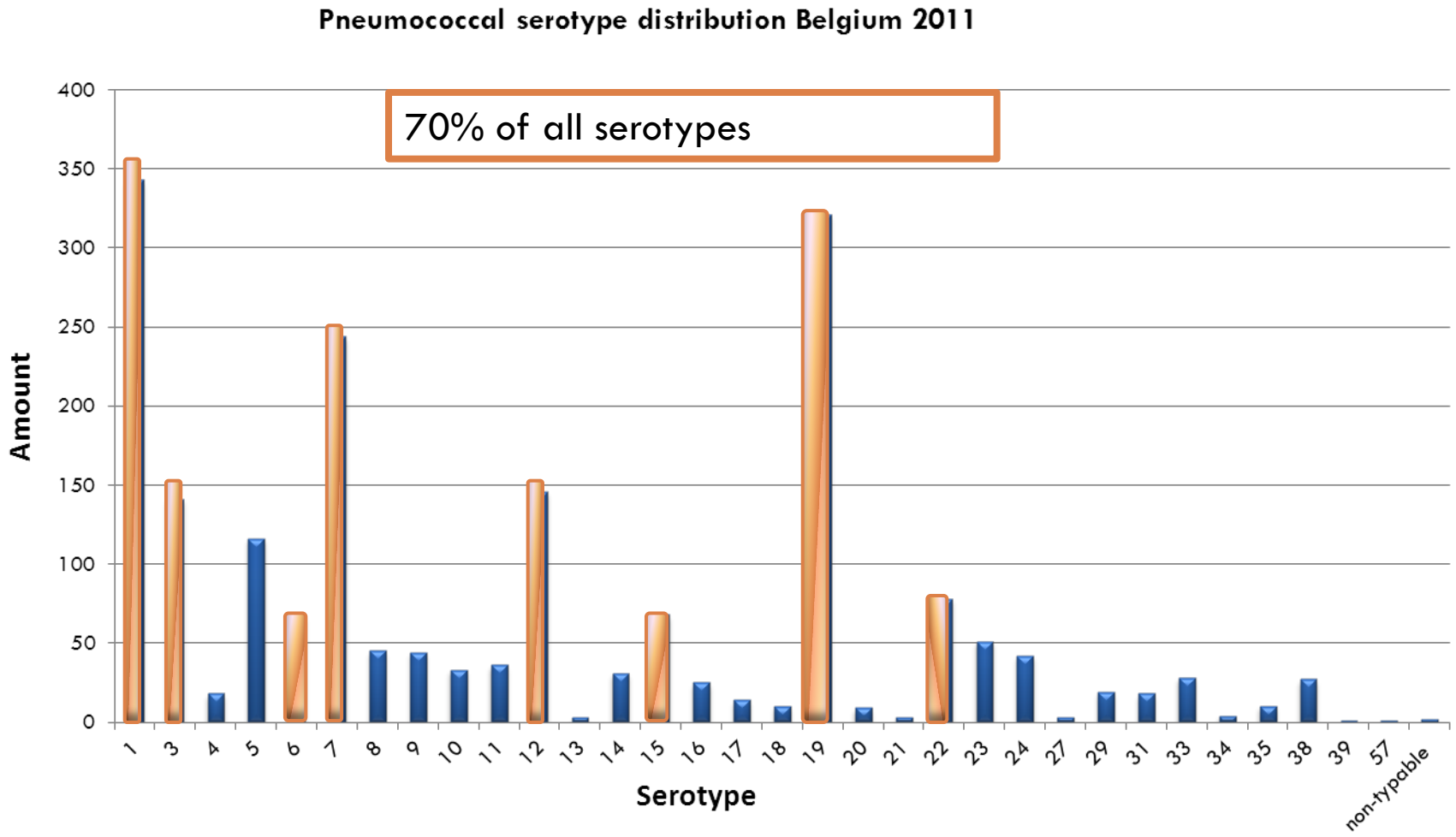
    ⇒ capsular swelling visualized under the microscope

  - **Limitations:**
    - High cost of antisera
    - Subjectivity of interpretation of results
    - Only on cultured microorganism



⇒ Need for new developments

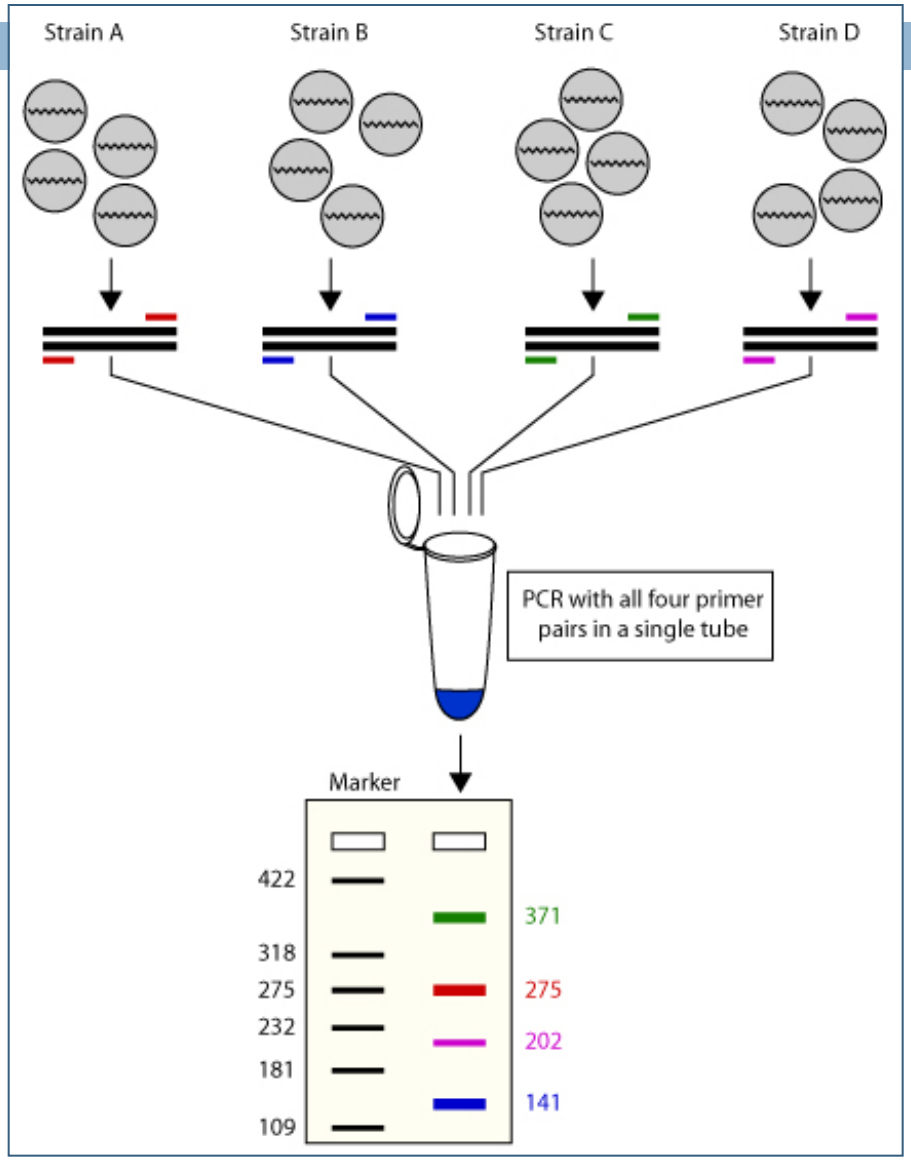
Pneumococcal serotype distribution in Belgium 2011



Primers CDC (update june 2011)

Primer Pair	Primer sequence (5'→3')	Product size (bp)
1-f	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	280
1-r	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	
3-f	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	371
3-r	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	
6A/B/C/D-f	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	250
6A/B/C/D-r	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	
7F/7A-f	TCC AAA CTA TTA CAG TGG GAA TTA CGG	599
7F/7A-f	ATA GGA ATT GAG ATT GCC AAA GCG AC	
12F/(12A/44/46)-f	GCA ACA AAC GGC GTG AAA GTA GTT G	376
12F/(12A/44/46)-r	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC	
15A/15F-f	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	434
15A/15F-r	GAT CTA GTG AAC GTA CTA TTC CAA AC	
19A-f	GAG AGA TTC ATA ATC TTG CAC TTA GCC A	566
19A-r	CAT AAT AGC TAC AAA TGA CTC ATC GCC	
22F/22A-f	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	643
22F/22A-f	CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC	

Conventional multiplex-sequential PCR reaction



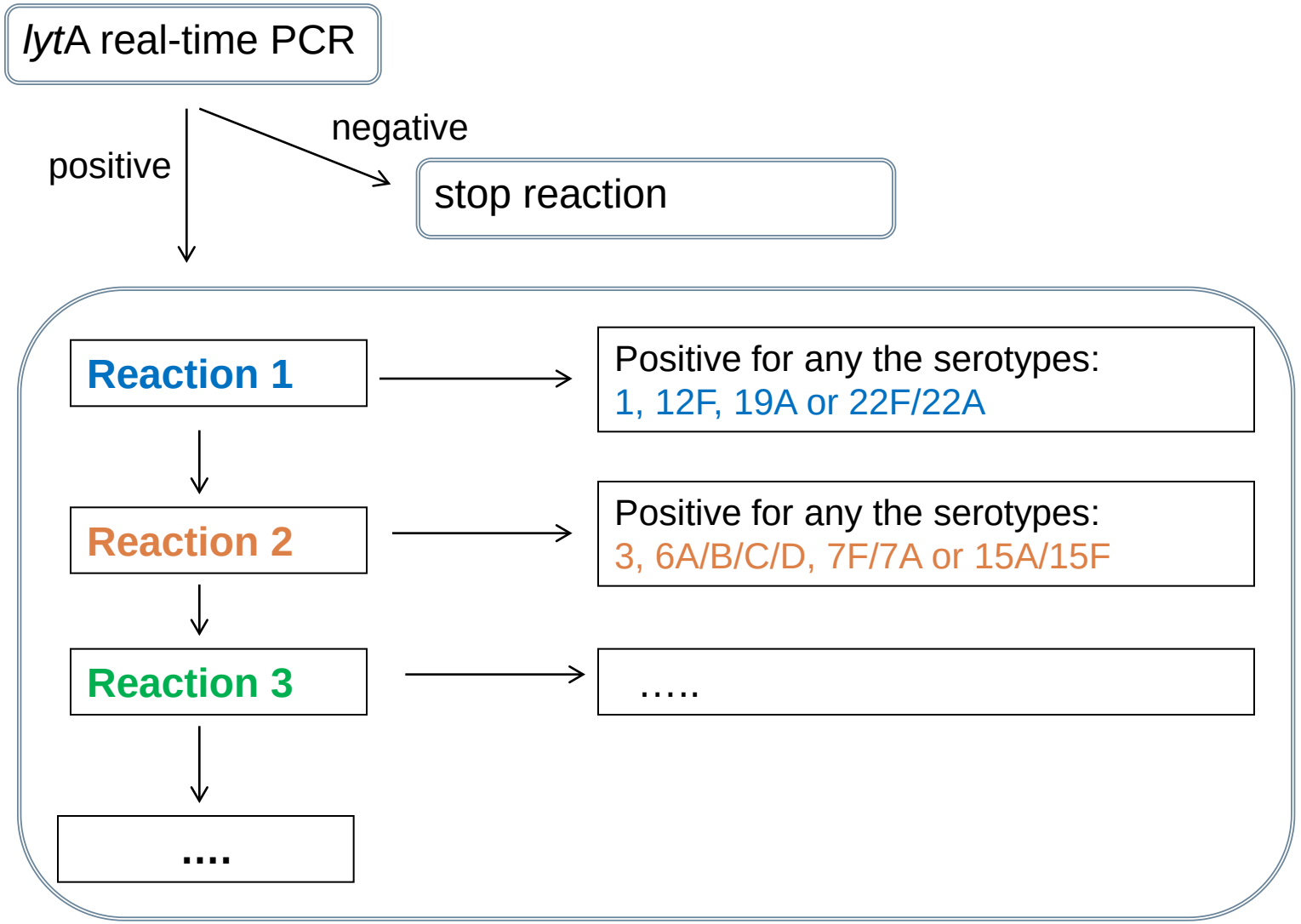
Multiplex-sequential PCR reaction

- **Two multiplex reactions** grouped together based on:
  - **Serotype distributions** among IPD in Belgium in the last two years
  - **Size of amplified products**: primers were combined in each reaction to yield differences of more than 70 bp in PCR fragment size for clear interpretation.

Reaction 1	
Serotype	Product size (bp)
1	280
12F	376
19A	566
22F/22A	643

Reaction 2	
Serotype	Product size (bp)
6 A/B/C/D	250
3	371
15A/15F	434
7F/7A	599

Multiplex-sequential PCR reaction



UZ LEUVEN: MS-PCR study protocol

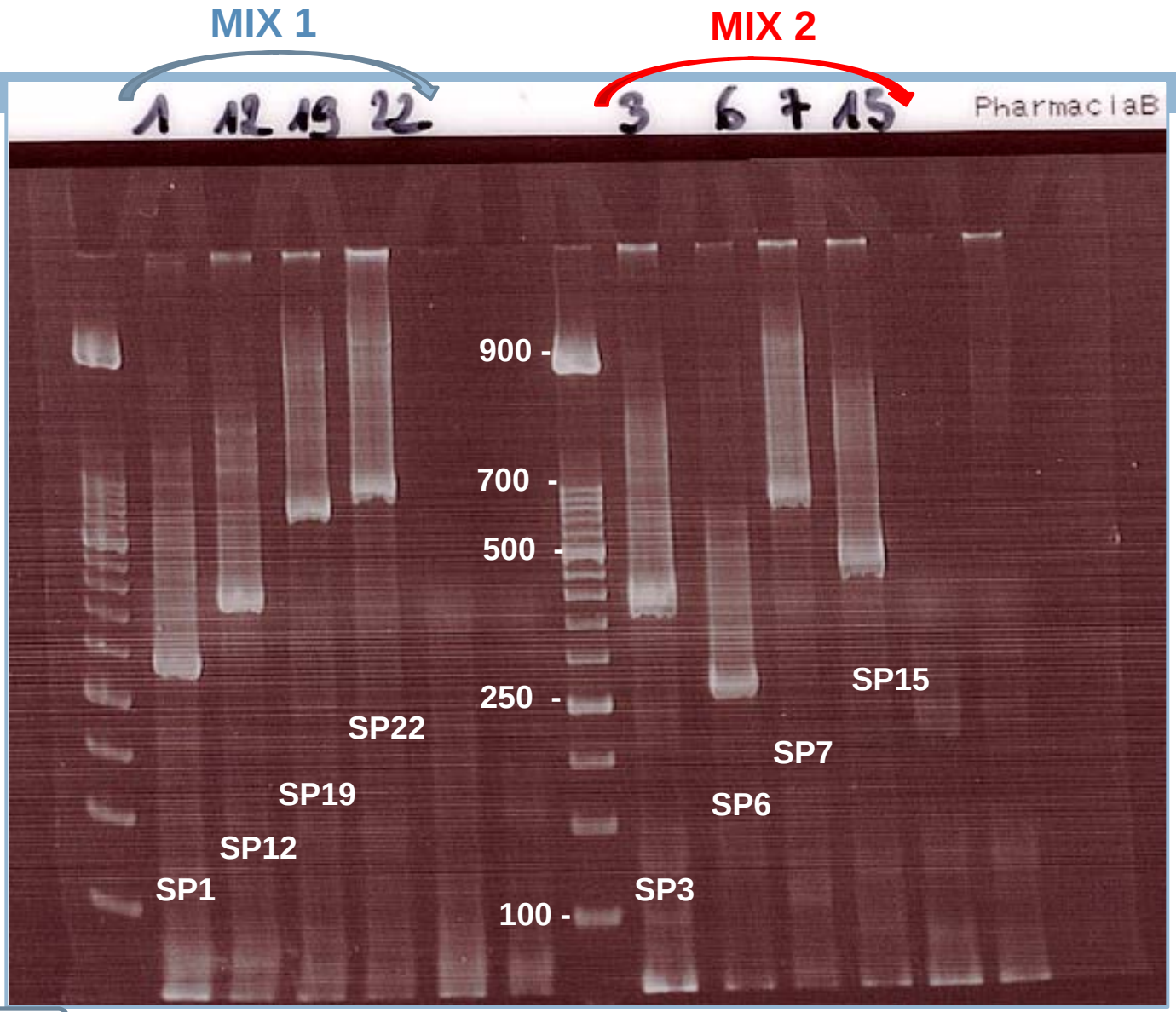
- **Material and methods:**
  - 60 invasive pneumococcal isolates of 8 different serotypes
    - UZ Leuven database
    - Preliminary capsular serotyping by Quellung reaction
  - Sequential multiplex reaction

REACTION 1: SEROTYPES 1-12F-19A-22F/A

REACTION 2: SEROTYPES 3-6ABCD-7F/A-15F/A

- **DNA extraction/PCR reaction:**
  - Extraction: Nuclisens easyMAG (Biomérieux)
  - PCR reaction: GeneAmp 9700.
  - Analysis: polyacrylamide gel electrophoresis at 200 V for 55 min.
  - Gels were stained with GELRED and gel images were recorded

MS-PCR on 60 bacterial isolates



Base pairs	280- 376- 566- 643	371- 250- 599- 434

Results MS-PCR: 60 bacterial isolates

- 60 invasive pneumococcal isolates of 8 different serotypes
- Preliminary capsular serotyping by Quellung reaction

Serotypes	No of isolates	% Concordance
1	8	100
3	8	100
6A/B/C/D	8	100
7F/A	8	100
12F	7	100
15F/A	7	**71 (5/7)**
19A	7	**86 (6/7)**
22F/A	7	100
	60	

Quellung subtyping

→ 15B, 15C

→ 19F

Literature: "serotyping on bacterial isolates"

Bacterial Isolates							
Author	**Year**	**Country**	**Isolates**	**No of samples**	**No of primer pairs**	**PCR reaction**	**Concordance**
Pai et al.	2006	USA	IPD (blood)	421	29	multiplex PCR	100%
Morais et al.	2007	Mozambique	IPD (blood, CSF)/AOM	153	29	multiplex PCR	97%
Dias et al.	2007	Brazil	IPD (blood, CSF, pleural fluid)	147	30	multiplex PCR	95%
Iraurgui et al.	2010	Spain	IPD	257	29	multiplex PCR	95,70%
Jourdain et al.	2011	Belgium	Nasopharyngeal aspirates	332	30	multiplex PCR	95,13%
Yun et al.	2011	Korea	All clinical specimens	77	30	multiplex PCR	98,70%
Miernyk et al.	2011	Alaska	Nasopharyngeal samples	1135	30	multiplex PCR	94%
Vickers et al.	2011	Ireland	AOM	144	11	multiplex PCR	96,90%

PART 4

Capsular serotyping of *S. pneumoniae* directly on pleural fluids

UZ LEUVEN: MS-PCR study protocol

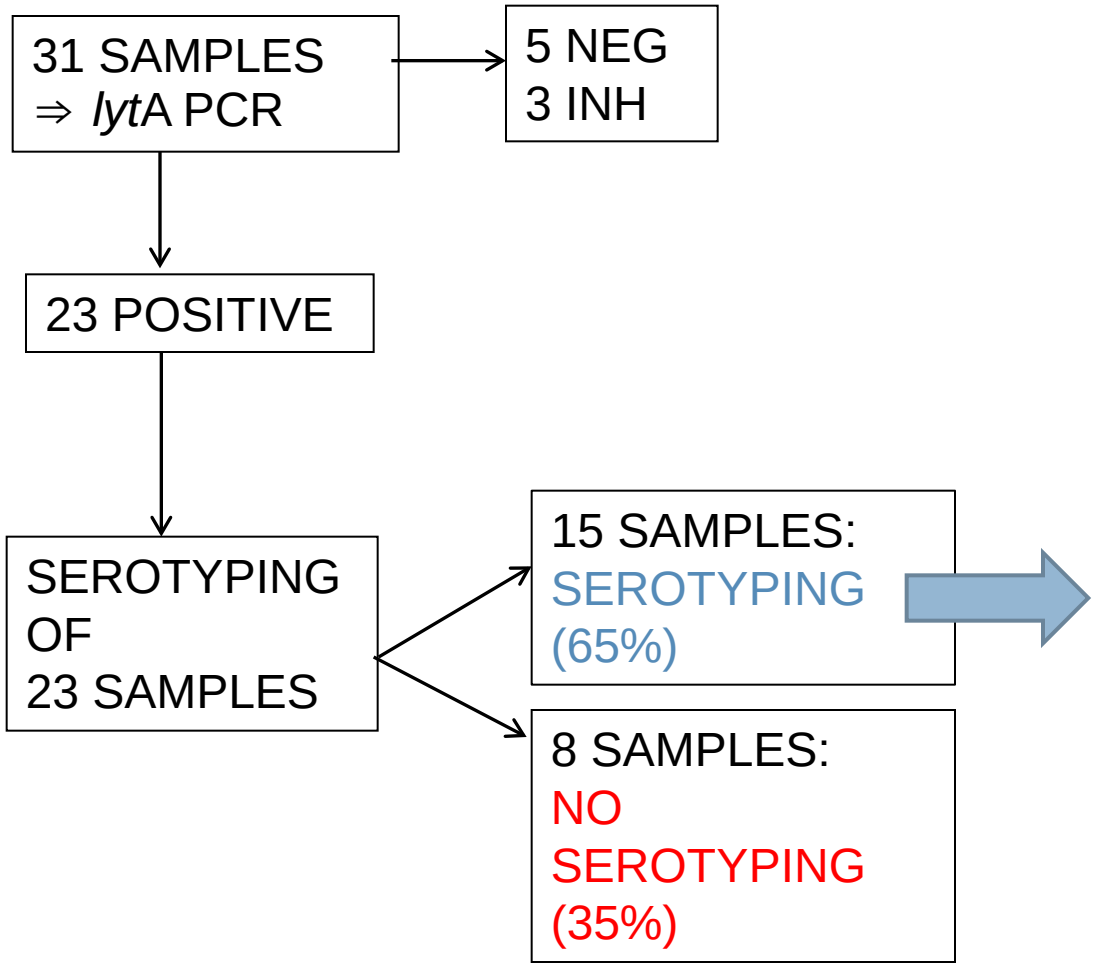
- **Material and methods:**
  - 31 pleural fluids
  - Sequential multiplex reaction

REACTION 1: SEROTYPES 1-12F-19A-22F/A

REACTION 2: SEROTYPES 3-6ABCD-7F/A-15F/A

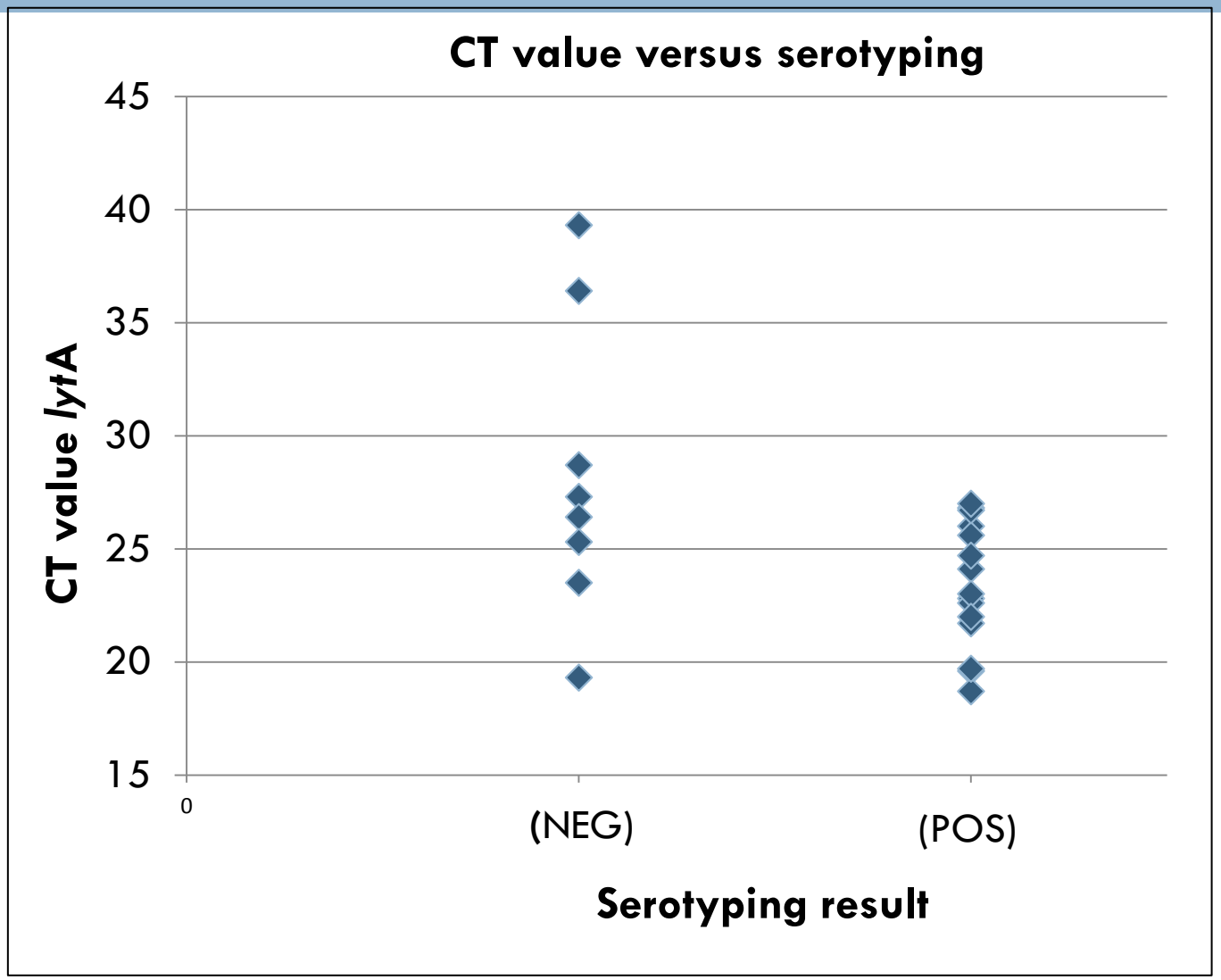
- **DNA extraction/PCR reaction:**
  - Extraction: Nuclisens easyMAG (Biomérieux)
  - PCR reaction:
    - Thermal cycling was performed in GeneAmp 9700.
  - Gel electrophoresis

MS- PCR results on 31 pleural fluid samples



Serotype	No samples	%
1	9	60
3	3	20
6A/B/C/D	0	0
7F/A	1	7
12F/(A)	0	0
15A/F	0	0
19A	2	13
22F/A	0	0

Ct value (lytA) versus serotyping result



Five most prevalent pneumococcal serotypes isolated from pleural effusions (Yu et al.)

TABLE 3. Summary of 11 published reports showing the five most prevalent pneumococcal serotypes

No. of samples	Collection date (yr)	Serotype (% of total) by ranking:					Sum (%)
		1	2	3	4	5	
10	1975–1978	1 (50)	3 (37.5)	7F (12.5)	X[a]	X	100
26	1993–1999	1 (50.0)	14 (15.4)	9V (15.4)	19F (3.8)	18C (3.8)	88.5
133	1993–2000	14 (29.1)	1 (24.4)	19 (9.0)	3 (8.4)	6 (8.4)	79.3
24	1996–2000	1 (45.8)	14 (12.5)	6B (8.3)	19F (8.3)	6A (4.2)	79.2
27	1997–2001	1 (53.1)	14 (15.6)	3 (9.4)	X	X	78.1[b]
11	1990–2002	1 (62.5)	4 (25.0)	5 (12.5)	X	X	100.0
35	2000–2003	14 (26.3)	3 (23.7)	1 (21.1)	6B (7.9)	9V (5.3)	84.2
30	2002–2004	19A (26.7)	1 (23.3)	14 (13.3)	3 (10)	23F (6.7)	100
27	2003–2004	1 (66.7)	4 (11.1)	3 (7.4)	7F (3.7)	9V (3.7)	92.6
50	2001–2005	1 (34.0)	3 (20.0)	19A (14.0)	19F (6.0)	7 (4.0)	78.0
51	2001–2007	1 (33.3)	3 (27.5)	19A (25.5)	7F (3.9)	17 (2.0)	92.2

[a] "X" indicates the absence of reported serotypes.
[b] Three samples (11.9%) were negative for the 13 serotypes tested.

Yu et al. *J Clin Microbiol* 2011 Feb;49(2):534-813

Literature 'capsular serotyping directly on clinical samples'

Clinical samples							
Author	**Year**	**Country**	**Isolates**	**No of samples**	**No of primer pairs**	**PCR reaction**	**Typeable**
Tarrago et al.	2008	Spain	Pleural fluid	88	35	RT-PCR	77,60%
Saha et al.	2008	Bangladesh	CSF + isolates	358	56	MS-PCR	94,00%
Antonio et al.	2009	Gambia	Nasopharyngeal samples	279	29	MS-PCR	65,90%
Njanpop et al.	2009	Burkina Faso/Togo	CSF + isolates	194	29	MS-PCR	79,30%
Carvalho et al.	2010	USA	Nasopharyngeal samples	100	40	broth enrichment + RT-PCR + MS-PCR	NA
Azzari et al.	2010	Italy	IPD samples	67	MS-PCR: 31 RT-PCR: 21	MS-PCR vs RT-PCR	M-PCR (64,2%) RT-PCR (91%)
Resti et al.	2010	Italy	Blood samples	80	21	RT-PCR	91,20%
Yu et al.	2011	USA	Pleural fluid	49	7	multiplex immunoassay + 19A PCR	73,50%
Strachan et al.	2011	Australia	IPD (pleural fluid)	43	NA	M-PCR RLB	65,1% typeable
Marchese et al.	2011	Italy	Blood samples	46	35	RT-PCR	80,4 % typeable

Azzari et al. MS-PCR versus RT-PCR

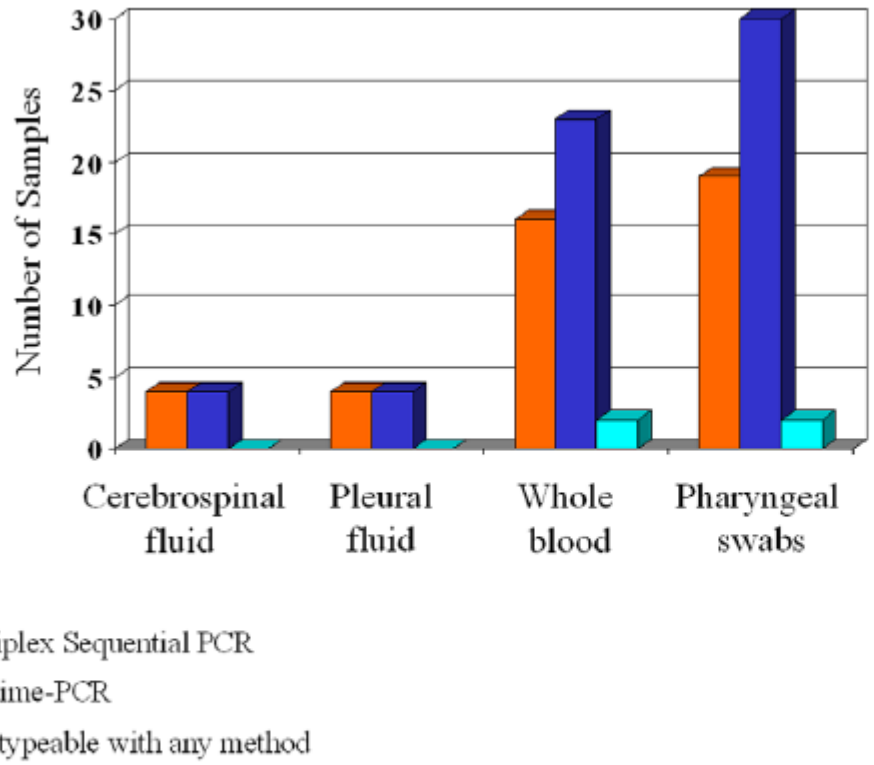


Figure 1. Number of typeable or non-typeable samples obtained from patients with culture-negative invasive pneumococcal infections as evidenced by Multiplex Sequential PCR or Realtime-PCR (sensitivity of Realtime vs Multiplex Sequential PCR p = 0.0004; 95%CL 1.98–17.05).
doi:10.1371/journal.pone.0009282.g001

Azzari et al. *PLoS One* 2010 Feb 19;5(2):e9282

Molecular serotyping

- **Major advantage:**
  - Type directly on culture-negative clinical samples
- **Serotype prevalence data:**
  - Possibility of replacement of PCV13 serotypes by nonvaccine serotype in the future
  - Introduction and development of new vaccines
- **Clinical importance:**
  - Serotype 19A: often multiresistant to antibiotics
  - Serotype 1: predilection for pleural space
  - Serotype 3:
    - Thicker polysaccharide capsule
    - Greater number of complications

Limitations

1. It's difficult to validate a PCR-based detection method on **culture-negative results.**

2. A sequential multiplex PCR cannot detect more than **29 serotypes** compared with 91 from the convential Quellung methods

3. The amount of **genetic variability** that exists among different isolates expressing the same serotype is unknown

4. Possibility to **misreading of the PCR gel.**

   $\Rightarrow$ the bands too close together

PART 5

Cost-effectiveness

Time-effectiveness

5. Time-effectiveness

Test time						
PROCEDURE	SECTION		Time (min)	Hands-on Time (min)	Total hands-on time (min)	Sample No
QUELLUNG	AVERAGE 8 test reactions		10	10	10	1
MS- PCR REACTION	EXTRACTION		90	50	200	15
	PCR	Preparation	90	90		
		PCR reaction	120	0		
	GELELECTROPHORESIS		120	60		

Hands-on time

Hands-on time for 1 sample (min)	
QUELLUNG	**10**
SEQUENTIAL MULTIPLEX PCR REACTION	**13**

Cost-effectiveness QUELLUNG

QUELLUNG REACTION (1 sample)			
Cost-effectiveness	**Cost (€)**	**Number**	**Total cost (€)**
1 test	0,45	8	3,6
Hands-on time	38/hour	0,17	6,5
		Total	**10,1**

Cost-effectiveness MS-PCR

	Multiplex PCR (1 reaction, 15 samples)			
		Cost (€)	**Number**	**Total (€)**
EXTRACTION	EasyMAG	7,86	15	117,9
PCR REACTION	Primers	0,02/primer	8	0,16
	PCR Express Mix	0,07/µL	375	26,25
	PCR Plate 7500	4,74/plate	0,16	0,79
	Tip 10 µL	0,05	15	0,75
	Tip 20 µL	0,05	10	0,5
	Tip 1000µL	0,07	4	0,28
GELELECTROPHORESIS	Gelelectrophoresis	2,31 +0,06/sample	15	3,21
HANDS-ON TIME	MLT	38€/hour	3,3	126,7
			Total (15)	**277**
			1 sample	**18**

Conclusion

- **Autolysin PCR** is more sensitive than culture for detection of *S. pneumoniae* in pleural fluids
- **BinaxNOW®:** rapid and sensitive method of diagnosis of pneumococcal empyema
- **MS-PCR** can be used for pneumococcal serotyping of most serotypes directly on clinical samples from culture-negative patients
- Pneumococcal serotyping was possible in 65% of *lyt*A positive pleural fluids, and **serotype 1** was the most frequent serotype.

To do's

1. Further **validation** of molecular serotyping on clinical isolates and on pleural fluids

    1. MS-PCR of serial **dilutions from pneumococcal DNA extracts** (based on Ct value *lyt*A PCR) to determine the threshold of detection

    2. Additional clinical isolates of **viridans group streptococci** should be assessed to ensure that no cross-reactivity occur (*lyt*A PCR and capsular serotyping)

2. The implementation of the next multiplex reaction based on the geographical epidemiology : **serotypes (5,8,9N and 24)**

Questions???